

PARTITION AFFINITY LIGAND ASSAY

Partitioning in aqueous two-phase systems
as a separation method in binding assays

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Torbjörn G. I. Ling

Department of Biochemistry
Chemical Centre
University of Lund

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Fil. kand. Torbjörn G. I. Ling (Mlm)

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Abstract Partitioning in aqueous two-phase systems has been used as separation step in binding assays. Aqueous two-phase systems are composed of solutions of two polymers or one polymer and a salt. They allow gentle conditions (biocompatibility) for separation. When the binding step is completed in a binding assay, a phase system is added to separate free and bound reactant, the principle being that bound reactant is found in one phase and free in the other. In practice, unilateral partitioning is generally not observed. In a direct binding assay there are two reactants, one of which is labelled. Binding can then be observed as a displacement of label from one phase to the other. In a competitive assay there are three reactants, e.g. antigen from a sample, labelled antigen and antibody. Antigen from the sample inhibits the binding of labelled antigen to the antibody. The assay is designed to give bound antigen in one phase and free in the other. In order to achieve efficient separation, one of the reactants can be modified to make it partition primarily to one phase. Modification can be effected by covalent attachment of a polymer, e.g. poly-(ethylene glycol) or dextran, or a low molecular weight compound.			
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This dissertation is based on the following papers, which are referred to by Roman numerals in the text.

I Mattiasson, B. and Ling, T. G. I. (1980)
Partition Affinity Ligand Assay (PALA). A new approach to binding assays
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II Mattiasson, B., Ling, T. G. I. and Ramstorp, M. (1981)
Application of Partition Affinity Ligand Assay (PALA) in a quick test for quantitation of Staphylococcus aureus bacterial cells
J. Immunol. Meth. 41, 105 - 114

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Immunological quantitation of Bacterial Cells Using a Partition Affinity Ligand Assay: A Model Study on the Quantitation of Streptococci B
Anal. Biochem. 122, 26 - 32

IV Ling, T. G. I. and Mattiasson, B. (1982)
A comparison between binding analyses performed by equilibrium dialysis and partitioning in aqueous two-phase systems exemplified by the binding of Cibacron blue to serum albumin
J. Chromatogr. 252, 159 - 166

V Ling, T. G. I. and Mattiasson, B. (1983)
A general study of the binding and separation in Partition Affinity Ligand Assay (PALA): Immunoassay of β_2 -microglobulin
J. Immunol. Meth., in press

VI Ling, T. G. I. and Mattiasson, B. (1983)
Poly-(ethylene glycol) and poly-(vinyl alcohol) substituted carbohydrate gels for "mild" hydrophobic chromatography
J. Chromatogr. 254, 83 - 89

INTRODUCTION

The process of living is promoted by the ability to communicate and interact, not only on a macroscopic scale but also at the molecular level. In the living cell virtually all biomolecules participate in some type of interaction. The study of binding between different molecules in biological systems has therefore been an important part of biochemistry. Biopolymers with binding properties, e.g. proteins and polynucleic acids, may interact with varying substances ranging from small molecules to large structures and whole cells. These interactions occur with very different binding strengths. For such a wide range of different reactants, it is not possible to use one standard method for studying all types of interactions. Thus, a large number of experimental procedures have been developed and still more emerge. There are, however, many problems inherent with the study of binding phenomena to which there are as yet no attractive solutions. Consequently, there is great interest in the development of new techniques.

In order to make qualitative or quantitative determinations of a particular substance, which is present in low concentration in a mixture of many other substances of basically similar chemical composition, very specific analytical methods must be used. To solve the problem of specificity, one can use the biochemical recognition systems used by nature; they include

interactions between e.g. antibody/antigen, receptor/ligand, lectin/carbohydrate, enzyme/inhibitor etc. The interaction between antibody and antigen is especially useful, since the immunological system, which is capable of recognising foreign structures (antigens), can be alerted in a test animal to produce antibodies with high specificity.

The early methods based on precipitation reactions between antigen and antibody were run with amounts that gave visible precipitates of the antigen-antibody-complex, either directly or after staining. The amounts of antigen and antibody required were, however, too high to allow measurements of substances present only in low amounts, as for example hormones. The advent of the radioimmunoassay (Berson et al, 1956; Yalow and Berson, 1959) opened the area of amplified immunoassays which have reached an enormous range of sensitivity. As a rule of thumb, a detection limit corresponding to 1 - 1000 ng antibody/ml can be expected, but assays with much higher sensitivity (1 pg/ml) have been reported (Wisdom, 1976; Ishikawa and Kato, 1978). It has even been reported that it is possible to measure the enzyme activity of a single antibody-enzyme complex, which has been allowed to work for a predetermined time (Rotman, 1973).

An amplified immunoassay utilises the binding of a molecule (antigen) to a protein with specific binding properties (antibody). One of the reactants is labelled, i.e. it is chemically linked with a radioactive isotope or an enzyme. In an competi-

tive immunoassay, an unknown amount of antigen from a sample is mixed with a fixed amount of labelled antigen and allowed to compete for a limiting number of binding sites on specific antibodies. A high concentration of antigen in the sample will inhibit the binding of labelled antigen to the antibodies and a low concentration will allow a high amount of labelled antigen to be bound. The amount of antigen in the sample can then be determined indirectly by measuring the amount of bound label. The terms antigen and antibody will be used for simplicity throughout the text, although the discussions on binding interactions are valid for different pairs of reactants as exemplified above.

In the first developed amplified immunoassay, the radioimmunoassay (Berson et al, 1956), insulin from a blood sample and insulin labelled with a radioactive iodine isotope were mixed with serum containing antibodies against insulin. Free and antibody bound material were then separated by electrophoresis. Two radioactive bands were found on the electro-chromatogram, one at the expected position for insulin and one in the zone where antibody is found.

The radioimmunoassay did, however, not come into more widespread use until 10 years later. Possible reasons for the delay may be that the general applicability of the method not was stressed in the original paper and that it involved a relatively slow and laborious separation procedure.

In this thesis the importance of the separation step in immunoassays and binding analysis is stressed. The separation is a crucial part of a binding assay and its performance will often directly affect the outcome of the assay.

SEPARATION OF FREE AND BOUND REACTANT

In order to determine the degree of binding, the amount of free and/or bound reactant must be measured. This generally requires a separation step. However, if the complex has some property that is different from both the reactants, this property can be used for the determination in homogenous solution. For example, chromophoric ligands may shift fluorescence or absorption spectrum when bound to a protein. Such probes have also been used as labels in immunoassays (Burd et al, 1977). Another approach is to design the antigen-enzyme conjugate so that the enzyme is inactivated when bound via the antigen to an antibody (Rubenstein et al, 1972). Immunoassays that do not need a separation step have been called separation independent immunoassays.

The above-mentioned assays are based on the existence of a special property of one of the entities in the mixture. They can therefore at present be regarded as special cases with limited general applicability.

In most cases there is thus no general method for avoiding the problem of separation. The choice of separation method for a specific task depends on the nature of the reactants. The separation can be based on physicochemical properties such as size, charge, solubility etc., but also on specific recognition mechanisms (precipitation with second antibody). A reactant can

also be assigned specific properties to facilitate its separation by, for example immobilisation on particles. Most of the separation methods known in biochemistry have also been used in binding assays. A listing of the more common ones is given in Tab. 1.

When different separation methods for binding assays are compared, the two criteria 'efficiency' and 'practicality' can be considered the most important (Chard, 1980). Maximum efficiency is then implied to occur when all reactant has been perfectly separated from the complex. In practice this cannot be achieved owing to two reasons. First, no separation method is, in this sense, perfect and separates all free and bound reactant. Secondly, even if this were the case, all binding phenomena are reversible and efficient removal of free reactant will cause dissociation of the complex (IV) and consequently some amount of free reactant will always be found together with bound. This fact has often been ascribed merely to "nonspecific binding" and "assay blank", which may be misleading (Chard, 1980).

The practicality of a separation method is determined by its speed, simplicity, applicability, reproducibility and cost (Chard, 1980). Adequate reproducibility is naturally a basic requirement in a binding assay. Simplicity is always an attractive characteristic of an experimental procedure and may keep the costs down. More important, however, is that fewer steps generally means higher reproducibility, since each step introduces a new parameter and thus a source of error. A high appli-

Tab. 1 SOME SEPARATION METHODS USED IN BINDING ASSAYS

Equilibrium dialysis

Ultrafiltration

Electrophoresis

starch gel

cellulose acetate

Gel filtration

column

batch

Precipitation, fractional

ammonium sulphate

poly-(ethylene glycol)

ethanol

dioxane

sodium sulfite

trichloroacetic acid

Precipitation, specific

second antibody

protein A

Solid-phase antibody

particles

polystyrene tubes/plates

Adsorption

charcoal

hydroxyapatite

silicate

References: Wisdom, 1976; Chard, 1980

cability means that one procedure can be used without modification to separate different pairs of reactants.

The most generally useful separation procedure in immunoassays may be the biospecific binding of the bound fraction by a second antibody. This mild procedure does not lead to significant dissociation of the antigen-antibody complex, and is specific for the complex so that free antigen is not included. Other methods of general applicability are precipitation of the bound fraction by salt or poly-(ethylene glycol). There is, however, a risk for coprecipitation of the free reactant, which is seen as high blank values in the assay. The amount of "free" reactant in the precipitate depends on the difference in solubility between the entities in the assay and varies greatly between different systems.

The discovery that antibodies can be immobilized to small particles or test tubes made of poly-styrene with retained binding capacity (Wide & Porath, 1967; Catt & Treagar, 1967) made it possible to greatly simplify the experimental procedure in immunoassays. Using this version, the separation of free and bound antigen takes place when the liquid content of the test tube is poured out. After a few washing steps, only the antigen bound to the antibody is left in the test tube.

This thesis concerns the partitioning in aqueous two-phase systems as the separation step in binding assays. A basic requirement in this method is that the conditions are chosen in such a

way that the entities that are to be separated partition to different phases. Aqueous two-phase partitioning has been used in biochemical work for separation of low molecular weight ligands, proteins and whole cells. Phase systems composed of PEG and dextran and salt have mostly been employed. This method, therefore, has a high applicability. The efficiency of the separation is dependent on the partitioning behaviour of the entities in the binding reaction and is discussed below.

PHASE SYSTEMS

When aqueous solutions of two polymers are mixed with each other the mixture may become turbid and after some time form a two-phase system, with each phase mainly consisting of one of the polymers. The formation of aqueous two-phase systems occurs for many combinations of polymers, as exemplified in Tab. 2. This phenomenon has long been known (Beijerinck, 1896), however, more thorough investigations of the properties of aqueous two-phase systems appeared much later (Dobry, 1948; Albertsson, 1958).

Aqueous two-phase systems allow very mild conditions for separation and are biocompatible, i.e., biological material such as cells and proteins can be partitioned without loss of biological activity (Albertsson, 1971). They are thus, in this respect, superior to conventional phase systems which contain organic solvents. Aqueous two-phase systems composed of two polymers have a very low interfacial tension ($0.0001 - 0.1 \text{ dyn/cm}^2$) and can furthermore be adapted to resemble physiological conditions by the addition of buffer, salt and carbohydrate.

The basis for separation in a two-phase system is the nonuniform distribution of substances between the phases. The distribution of a substance is characterized by the partition coef-

Tab. 2 SOME POLYMERS GIVING TWO-PHASE SYSTEMS

	PPG	PEG	PVA	PVP	MC	HPD	Ficoll
PEG	*						
PVA	*	*					
PVP	*	*					
MC	*		*	*			
HPD	*	*	*		*		
Ficoll	*	*					
Dextran	*	*	*	*	*	*	*

Reported incompatibility between polymers is indicated with an asterisk. This is equivalent to the possibility of formation of aqueous two-phase system. Data from Albertsson (1971).

Abbreviations:

PPG	poly-(propylene glycol)
PEG	poly-(ethylene glycol)
PVA	poly-(vinyl alcohol)
PVP	poly-(vinyl pyrrolidon)
MC	methylcellulose
HPD	hydroxypropyldextran
Ficoll	poly-sucrose
Dextran	poly-glucose

ficient, K_{part} , defined as the ratio of the concentrations in the top and bottom phase, respectively:

$$K_{\text{part}} = \frac{C_{\text{top}}}{C_{\text{bottom}}}$$

Small molecules, biological macromolecules, cell organelles and cells distribute in aqueous two-phase systems according to their surface properties. The distribution can be changed by changing the composition of the phase system. The partition coefficient is composed of several factors that reflect the partitioning behaviour due to electrical, hydrophobic, hydrophilic and conformational effects (Albertsson, 1977).

When particulate matter is partitioned, the situation is more complicated because of possible adsorption at the interface between the two bulk phases.

A typical phase diagram for a two-phase system is shown in Fig. 1. The curved line in this diagram is called the binodial curve. Mixtures of polymers in concentrations above the curve, e.g., point P, result in phase systems, whereas polymer mixtures below the binodial curve, e.g., point S, give a homogeneous solution. In a phase system with a total composition as indicated by P, the two phases have the compositions Q and R, respectively. The line QR is called tie-line. A linear relationship has been found between the logarithm of the interfacial tension and the length of the tie-line (Albertsson,

1971). The tie-line also gives information about the volume ratio, or, more exact, the mass ratio between the two phases. If the phases are denoted Q and R the expression can be written as $\text{Mass}_Q / \text{Mass}_R = \text{PR} / \text{PQ}$, where PR and PQ are the distances between the indicated points on the tie-line.

Differences in molecular weight of the phase components can markedly influence the separating capacity of the system. Lower concentrations are sufficient for phase separation when polymers of a higher molecular weight are used.

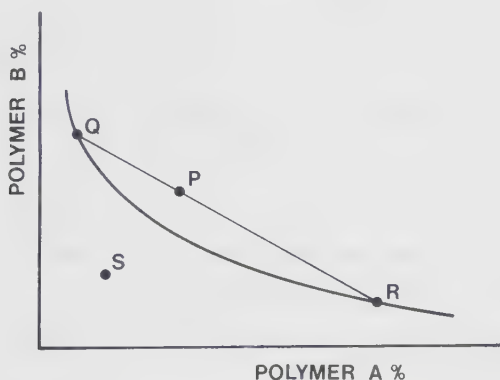


Fig. 1. Phase diagram for two polymers, A and B, mixed in water. Phase separation occurs if the polymers are present in enough concentrations. Compositions giving phase systems are represented by points above the binodial curve such as P, while compositions giving homogenous solutions are represented by points below the curve. The line QR is called a tie-line and the points Q and R represent the compositions of the phases in a two-phase system with a total composition P.

The time required for phase separation varies greatly between different systems and depends on the viscosity of the polymer solutions and on the distance from the binodial curve. In analytical procedures quick and reproducible separation is a basic requirement.

Buffers and other salts are often added to phase systems. When a salt consists of ions that have different affinities for the two phases, the ions will distribute unevenly at the interface and thus cause a potential across it. This interfacial potential will affect the partitioning of charged entities, especially those with a high net charge, such as cells and other biological particles. The change of partitioning is proportional to both the net charge and the interfacial potential (Albertsson, 1977).

Another way to partition compounds according to their charge is to include polymers with charged groups in the phase system (Johansson, 1970).

A more selective way of changing the partitioning is to utilize biospecific interactions between a low molecular weight ligand and a protein. The ligand that is to be used is coupled to one of the polymers used in the phase system. In this way the partitioning of the ligand is secured. If the system is properly designed, the ligand will have the same partitioning when bound to its reactant and thus be able to move it. This method has

been called affinity partition (Shanbhag and Johansson, 1974; Flanagan et al, 1976; Hubert et al, 1976).

The parameters mentioned above can be adjusted in a system to give the desired partitioning of the reactants, and thus the separation effect. If the reactants can be only partly separated in a phase system, the procedure can be expanded to a multi-step procedure, counter-current distribution (CCD), to give better separation.

In this thesis two types of phase systems are employed: poly(ethylene glycol)/magnesium sulphate, in which separation takes 5 - 15 minutes, and poly(ethylene glycol)/dextran, in which separation takes 5 - 60 minutes. The PEG-rich phase is the top phase in both the phase systems. When the determination of binding was based on direct photometric measurements of one of the phases in the phase system, that phase had to be in a clear state. This could be achieved by varying the ratio between the volumes of the two phases since the smaller phase generally is adequately clarified.

CHARACTERISTICS OF PARTITION AFFINITY LIGAND ASSAY (PALA)

The principle for studying direct binding reactions in aqueous two-phase systems is schematically illustrated in Fig. 2. The composition of the phase system is chosen so that the antigen partitions to one phase (Fig. 2a) and the antibody to the other (Fig. 2b). The degree of binding can then be observed as a displacement of antigen when antibodies are added to the system and antigen is moved to the phase to which the antibodies par-

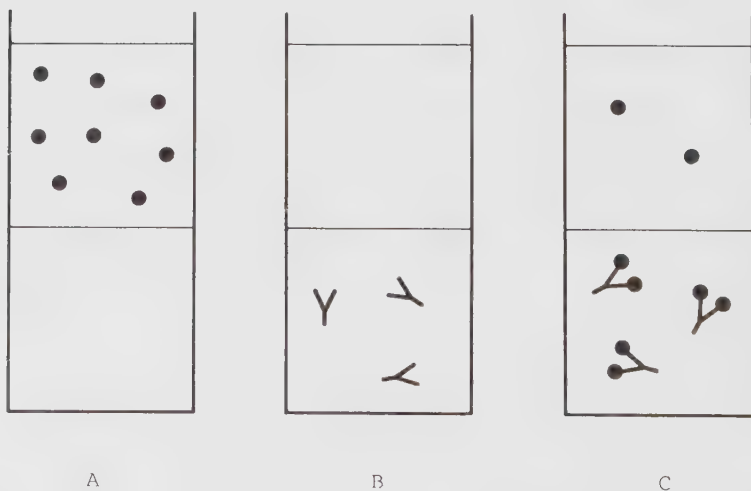


Fig. 2. Schematic representation of a direct binding assay between antigen and antibody. (A) The antigen partitions to the top phase and (B) the antibody partitions to the bottom phase. (C) Binding is observed as a displacement of antigen from the top phase to the bottom phase.

tition (Fig. 2c). In practice the two reactants do not have completely unilateral partitioning, but the two partition coefficients give the end-points for the standard curve.

In a competitive binding assay, Fig. 3, native antigen (from the sample) competes with a fixed amount of labelled antigen for the limiting amount of binding sites on the antibodies. The labelled antigen and the antibody must partition, more or less, to different phases. The partitioning of the unlabelled, native antigen is of less importance.

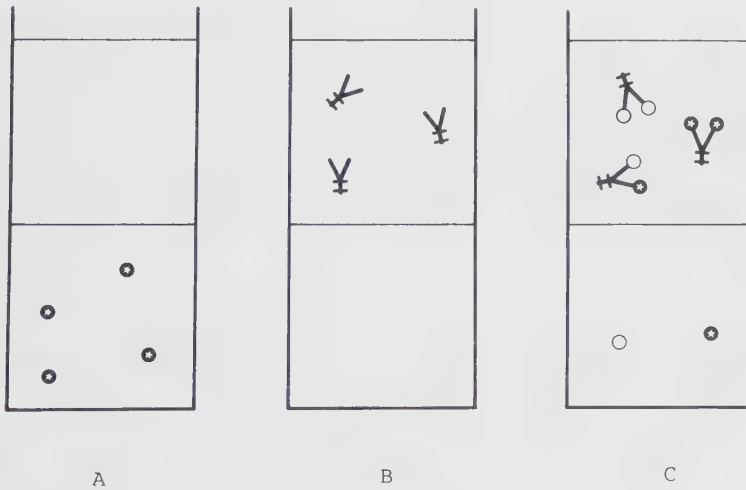


Fig. 3. Schematic representation of a competitive binding assay. (A) Labelled antigen partitions to the bottom phase. (B) PEG-modified antibody partitions to the top phase. (C) In the competitive binding situation, antigen from a sample and labelled antigen are mixed with a limiting number of antibodies. After separation, bound antigen is found in the top phase and free in the bottom phase.

As already mentioned, the separation step in a binding assay must be highly reproducible and preferably also fast. This means that when phase systems are used for separation, the effect of salts and other substances that influence partitioning should be minimized. It also means that long multi-step procedures (CCD) should be avoided. Both these requirements imply the use of an effective separation procedure and can be met by employing an antibody with strongly asymmetric partitioning and thus low sensitivity for minor changes in the composition of the phase system. This method has been called Partition Affinity Ligand Assay (PALA). Proper partitioning of the antibody can be accomplished by choosing a proper phase system (Mattiasson, 1980; Mattiasson and Eriksson, 1982), or, if necessary, by modifying the surface structure of the antibody (I, II, V) in order to make it partition exclusively to one of the phases. Such modified binding structures have been called separator molecules (II).

In general, if the reactants in an assay have similar partitioning behaviour, one of them has to be modified, however, not necessarily the antibody or binding structure. In the competitive assay for streptococcal cells (II) the antibodies were bound either to the native cells from the sample or to the modified cells. A similar approach was used in the assay for yeast cells (Mattiasson et al, 1982). Modified cells have in analogy been called separator cells.

When an immunoassay is designed, the partitioning of the labelled antigen is determined first of all. It should have at least moderately asymmetric partitioning, if necessary this can be accomplished by changing the composition of the phase system. Then the partitioning of the antibody is determined. If the antibody partitions to the same phase as the labelled antigen it must, as a rule, be modified to make it partition to the other phase. The third reactant, the native antigen from the sample, may have a partitioning coefficient that is different from that of the labelled antigen. The partitioning of the native antigen is, however, generally not very critical as long as it is not too extreme.

Modification has mostly been effected by covalent attachment of a polymer, e.g. PEG, to make the substance of choice partition to the PEG-rich top phase (I). Dextran in the form of the single polymer (Mattiasson and Ling, 1983), or crosslinked in Sephadex particles (Mattiasson, 1980), enable the substance to partition to the salt-rich bottom phase. Organic moieties such as phenyl groups and short alkane chains (octyl, dodecyl) have also been used for modification (H. Nilsson, personal communication). The formation of an antibody-enzyme conjugate can also be regarded as a modification of the antibody, since it is possible to change its partitioning in this way (III).

When molecules are modified by coupling to particles, as for example Sephadex beads, the density of the particle must be considered. In most phase systems there is a significant dif-

ference in density between the phases, and the particles can therefore be distributed according to their bouyancy.

When an antibody is modified by attachment of PEG, its surface properties are altered giving it a higher partition coefficient. However, the PEG molecules may sterically hinder binding reactions. Highly modified antibodies have a significantly decreased binding capacity. If a PEG-modified antibody is to be used in an assay, it should be able to partition predominantly to the top phase and as well as retain a fair amount of its binding capacity. Each batch of modified antibodies has, therefore, been tested for their ability to bind antigen and move it to the top phase (I, II, III, V). This ability has been called transport capacity. For most types of binding structures, the degree of modification has an optimum. A further discussion on the properties of native and modified reactants is found in the chapter "Immunoassays".

The basic PALA procedure is presented in Tab. 3. Besides the general procedure, three alternatives for simplifications are given. In alternatives B, the whole assay - binding and separation is carried out in one and the same vessel. After the incubation time is finished, only one addition of phase components and enzyme substrates is made and the cuvette is left for 5 - 60 minutes before the enzyme activity is determined. A possible drawback with this version may be that some two-phase systems give turbid phases that should be diluted before

Tab. 3 SCHEMATIC PROCEDURE FOR PALA

A. General procedure for isotope- and enzyme-labelled assays

1. Mix the reactants, (e.g. antigen, labelled antigen and antibody) and allow an adequate incubation time for the interaction studied (5 min - several hours).
2. Add a premixed phase system and allow sufficient time for the separation process (5 - 30 min).
3. Take out aliquots for determination of the amount of label in each phase (or one phase only).

B. Simplified procedure for enzyme-labelled assays

1. As in A:1, but a cuvette is used to facilitate the subsequent determination of the amount of label.
2. Add the phase components containing the enzyme substrates. After separation, the cuvette is placed in a photometer for determination of enzyme activity in one of the phases.

C. Simplified procedure for isotope-labelled assays

1. As in B:1, but a gamma-counter tube is used instead of the cuvette
2. As in B:2, but after separation, the tube is placed in a gamma-counter for determination of radioactivity in one of the phases.

D. One-step procedure for enzyme-labelled assays

1. As in B:1 and B:2, but the incubation time in B:1 is omitted so that the phase system is added with the reactants.

measurement in the photometer and thus can only be used with the general procedure.

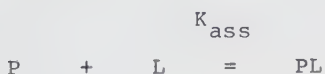
Alternative C is similar to B, but is used with gamma emitting isotopes. In this version, one of the phases is large enough to displace the other from the gamma-detectors counting zone so that primarily the radioactivity in one phase only is measured (Mattiasson et al, 1983).

Alternative D is even more shortened and simplified. Both the sensitivity and the reproducibility are generally decreased when the incubation time is shortened. Different binding reactions require, however, very different incubation times in order to reach equilibrium, and this time dependence must be determined for the actual reactants. The principal application of this alternative would be in rapid semi-quantitative assays. In these assays, the measurement in the photometer may be replaced by an visual examination. Since the colour intensities in the two phases can be compared with each other, a fairly accurate relative scale can be obtained. Further, the level where both phases are equally coloured may be adjusted to coincide with a critical concentration level. Any of the alternatives can, owing to the low number of steps, easily be automated.

BINDING ANALYSIS

As pointed out previously, the art of performing binding analyses is often the art of separation of free and bound antigen. Most of the separation methods used in biochemistry have also been used in binding analyses. A good separation method must be able to separate the reactants without perturbation of the binding reaction. This statement may sound superfluous and self-explanative, but the perturbation effect is not always easily avoided and its consequences may be difficult to establish. After the degree of binding has been determined in an analysis, the data may be used for calculation of the association constant or stoichiometry of the reaction.

The interaction between a protein, P, and a ligand, L, produces a complex, PL, as represented below:



The association constant, K_{ass} , tells how much complex is formed at equilibrium:

$$K_{\text{ass}} = C_{\text{PL}} / C_{\text{P}} * C_{\text{L}} \quad (1)$$

If the complex gets completely isolated during the separation process, the complex will dissociate slightly and after some time establish a new equilibrium. In practice, complete separation does not occur. However, many methods are designed to remove most of the free reactants, which then constitutes a risk for dissociation. Another factor that governs the dissociation is, of course, the association constant, K_{ass} . However, even if the complex is not thermodynamically stable, it may be kinetically stable if the dissociation is slow.

It is thus evident that a separation method that is to be used in analytical procedures must be highly reproducible and may have to be precisely timed (Eriksson et al, 1981).

Another problem that may occur involves interactions between the reactants and the material used in the separation process. Many binding reactions are studied in a very low concentration range (pM - μ M) and are thus very sensitive to perturbations, e.g. adsorption phenomena. Adsorption to the plastic parts in a reaction vessel, to ultrafiltration membranes (IV), gel filtration media, etc, may occur.

The commonly used method of equilibrium dialysis thus has an inherent weak point. This method is often used for determining binding between a protein and a low molecular weight ligand. Many of the ligands studied are of a rather hydrophobic nature, have a low solubility and are prone to adsorb to the dialysis membrane. Such adsorption may be strong enough to completely

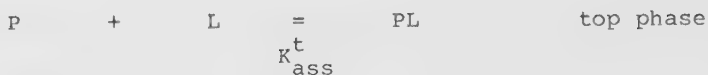
distort the outcome of a binding analysis (IV, Shanbhag et al, 1973).

Problems of this kind are not discussed very often in the literature. A survey on papers with Scatchard plots based on equilibrium dialysis revealed many curves of doubtful nature.

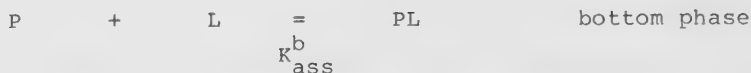
The separation of bound and free reactant in aqueous two-phase systems has, in this respect, two advantages:

1. The degree of separation can be controlled. It can be kept reproducible between different batches of phase systems (Albertsson, 1971) and is not time-dependent after equilibrium has been reached.
2. There are generally no problems with adsorption of biomolecules. However, particles and very large protein aggregates may be adsorbed to the interface.

When a binding analysis is run in a phase system, the following scheme is applicable. The superscript *t* denotes the top phase and *b* the bottom phase.



$$K_{part,P} \quad K_{part,L} \quad K_{part,PL}$$



The concentration of ligand in each phase can be expressed as:

$$C_{L,tot}^t = C_L^t + C_{PL}^t \quad (2)$$

$$C_{L,tot}^b = C_L^b + C_{PL}^b \quad (3)$$

For some interactions, e.g. between two proteins, the presence of the polymers that compose the phase system may slightly displace the equilibrium. This factor can be kept under control by making runs at different polymer concentrations and then extrapolating to zero concentration (Middaugh and Lawson, 1980).

Binding analyses in aqueous two-phase systems have been made previously (Winlund & Chamberlin, 1971; Albertsson, 1971; Shanbhag et al, 1973), but always with the prerequisite that the binding structure - protein or polynucleic acid - partitions exclusively to one phase. However, the exact analytical expressions for the partitioning behaviour of two interacting molecular species, A and B, have been derived (Albertson, 1978). Using the same symbols as above, they are:

$$K_{\text{part},A}^t K_{\text{part},B} (C_{A,\text{tot}}^b - C_{B,\text{tot}}^b - (C_{A,\text{tot}}^t - C_{B,\text{tot}}^t) / K_{\text{part},B}) * Q1$$

$$K_{\text{ass}}^t = \frac{(K_{\text{part},B} - K_{\text{part},A}) (C_{A,\text{tot}}^t / K_{\text{part},A} - C_{B,\text{tot}}^t / K_{\text{part},B} - C_{A,\text{tot}}^b + T)}{}$$

where

$$Q1 = C_{A,\text{tot}}^b - C_{B,\text{tot}}^b - (C_{A,\text{tot}}^t - C_{B,\text{tot}}^t) / K_{\text{part},A}$$

and

$$T = C_{B,\text{tot}}^b$$

$$(C_{A,\text{tot}}^t - C_{B,\text{tot}}^t - K_{\text{part},B} (C_{A,\text{tot}}^b - C_{B,\text{tot}}^b)) * Q2$$

$$K_{\text{ass}}^b = \frac{(K_{\text{part},A} - K_{\text{part},B}) (K_{\text{part},A} C_{A,\text{tot}}^b - K_{\text{part},B} C_{B,\text{tot}}^b - C_{A,\text{tot}}^t + U)}{}$$

where

$$Q2 = C_{A,\text{tot}}^t - C_{B,\text{tot}}^t - K_{\text{part},A} (C_{A,\text{tot}}^b - C_{B,\text{tot}}^b)$$

and

$$U = C_{B,\text{tot}}^t$$

$$K_{\text{part},AB} = K_{\text{part},A} K_{\text{part},B} \frac{C_{B,\text{tot}}^t / K_{\text{part},B} - C_{A,\text{tot}}^t / K_{\text{part},A} + C_{A,\text{tot}}^b - V}{K_{\text{part},A} C_{A,\text{tot}}^b - K_{\text{part},B} C_{B,\text{tot}}^b - C_{A,\text{tot}}^t + W}$$

where

$$V = C_{B,\text{tot}}^b$$

and

$$W = C_{B,\text{tot}}^t$$

This thesis presents another approach with evaluation of results from binding analyses where neither of the reactants have unilateral partitioning. It is, however, required that the reactants are primarily found in different phases and that their partition coefficients are known. After the total amount of label in each phase is determined the evaluation can start.

The following set of equations is used and solved by iteration (IV, V). As an initial approximation, $C_{L,tot}^t$ is used instead of C_L^t in eqn 4.

$$C_L^b = C_L^t / K_{part,L} \quad (4)$$

$$C_{PL}^b = C_{L,tot}^b - C_L^b \quad (5)$$

$$C_{PL}^t = C_{PL}^b * K_{part,PL} \quad (6)$$

$$C_L^t = C_{L,tot}^t - C_{PL}^t \quad (7)$$

This method has been used to evaluate binding analyses of the interactions between serum albumin and Cibacron blue (IV) and an immunoassay of β_2 -microglobulin (V). A more detailed description on the evaluation procedure has been published (IV, V). The method as presented here and in papers IV and V is only a basic concept and can be further developed for calculations involving more complicated binding situations.

IMMUNOASSAYS

When a competitive immunoassay is set up, the partitioning of the labelled antigen is determined first of all. It should have at least moderately asymmetric partitioning, if necessary this can be accomplished by changing the composition of the phase system. Then the partitioning of the antibody is determined. If the antibody partitions to the same phase as the labelled antigen, it must, as a rule, be modified to make it partition to the other phase. The third reactant, the native antigen from the sample, may have a partitioning coefficient that is different from that of the labelled antigen. The partitioning of the native antigen is, however, generally not very critical as long as it is not too extreme.

In the competitive assay as described above, there are two reactants that can be modified to give adequate partitioning - the labelled antigen and the antibody. When an enzyme is used as a label, the labelling can be regarded as a modification (III), provided that an enzyme with suitable partitioning properties can be found. Furthermore, the enzyme can be modified prior to conjugation with the antigen. Horseradish peroxidase which has been used as enzyme label in different assays (I, II, V) contains 18% carbohydrates and is thus naturally modified to partition to the bottom phase. It can, however, be modified

with various compounds to alter its partitioning (Mattiasson and Ling, 1983).

Depending on the partitioning behaviour of the labelled antigen, the antibody should then be modified, if necessary.

In order to be able to assign suitable partitioning properties to the antibody, there must be methods both for making it partition to the top and the bottom phases. If the labelled antigen partitions primarily to the top phase, the antibody should partition to the bottom phase, and may then be used in its native form (II, Mattiasson, 1980; Mattiasson and Eriksson, 1982), or modified by coupling to Sephadex particles (Mattiasson, 1980) or dextran (Mattiasson and Ling, 1983). Sephadex and other gel particles may also have a density that is large enough to prevent them from entering the top phase.

If the antibodies should partition to the top phase, they may be modified with PEG (I,V), with other polymers that can constitute the top phase (e.g. poly-(propylene glycol, poly(vinyl alcohol), or with low molecular weight compounds of hydrophobic nature (e.g. benzylamine, octylamine, phosphatidylserine) (Mattiasson and Ling, 1983). If the antibody is labelled with an enzyme, the conjugate may have a proper partitioning so that further modification is unnecessary (III). Different antibodies have different structures in the antigen binding site. It is consequently difficult, if not impossible, to find a modification procedure that only affects parts of the molecule that are

remote from the binding site, since any amino acid may be found in it. Furthermore, modification with PEG may cause steric hindrance in the interaction with high molecular weight antigens, even if the binding site and its vicinity is intact.

This dilemma was solved by coupling the antibody to a previously modified molecule or particle - a second separator (V).

In the discussion so far it is assumed that it is possible to find conditions that allow the antibody to move the labelled antigen from one phase to the other. If, however, the antigen is considerably bigger than the antibody, the complex may be found in the same phase as the free antigen. In this case it may be advantageous to use a direct binding assay, where labelled antibody binds to native antigen.

In this type of assay, the more antigen that is added, the more antibodies are bound and moved over the interface. It is also possible to use a competitive assay provided that the two antigenic species have sufficiently different partitioning properties. This approach has been successfully employed in three assays (II, III, Mattiasson et al, 1982).

Assays of cells may be run in the same way as other immunoassays. The partitioning of a substance in a two-phase system is dependent on its surface properties rather than its size. Consequently, cell organelles and whole cells can be partitioned following the same basic rules as macromolecules. There

is, however, one phenomenon that is special to cells - the adsorption to the interface. Cells may partition between one phase and the interface and not be present in the other bulk phase at all. This property should not be considered a disadvantage, since it is possible, from a practical point of view, to compare it with the case of unilateral distribution, as discussed above. In the direct assay of streptococci, the cells were found in the bottom phase and at the interface and the free labelled antibodies in the top phase. Aliquots were taken only from the top phase for determination of the amount of label. The distribution of labelled antibody between the bottom phase and the interface was then immaterial.

By using the described technique, the field of immunoassays can, for the first time, be expanded to cover assays of whole cells. Previous methods have generally relied on extraction of antigenic material from the cell surface. A listing of quantitative immunoassays is given in Tab. 4. More examples can be found in Mattiasson and Ling (1982) and Mattiasson and Ling (1983).

Tab. 4 PARTITION AFFINITY LIGAND ASSAY - SYSTEMS STUDIED

Antigen	Separator	Other reactant	Label Type	-- Time I (min) S	Concentration range	Ref
Glucose	PEG-ConA	Glucose	HRP Comp	10-30 10	20-1000 μ M	I
Staphylococci	(Staph)	IgG*	I Dir	30 120	$10^6 - 10^7$	II
Staphylococci	PEG-Staph	IgG*	I Comp	30 120	$10^5 - 10^7$	II
Streptococci	(Ab*)	Ab*	HRP Dir	30,60 60	2500-100000	III
Streptococci	PEG-Strep	Ab*	I Comp	30,60 60	25000-100000	III
β_2 microglob.	PEG-Ab	β_{2m}^*	I Comp	20 15	(3-96 μ g/l)	IV
β_2 microglob.	PEG-Staph	β_{2m}^*/Ab	I Comp	20 15	3 - 96 μ g/l	IV
Digoxin	(Ab)	Ab/Digoxin*	I Comp	5 5	1 - 8 nM	a)
T3	(Ab)	Ab/T3*	I Comp	30 30	1 - 6 nM	b)

Explanations: Label: HRP = Horseradish peroxidase; I = Iodine-125

* denotes a labelled reactant

Type: Comp = Competitive assay; Dir = Direct binding assay

Time: I = Incubation of the reactants (at room temp.); S = Separation

a) Mattiasson, 1980 b) Mattiasson and Eriksson, 1982

PURIFICATION OF MODIFIED SEPARATORS

It has been found that modified separator molecules sometimes do not have enough transport capacity to work well in an assay, depending on, for example, the presence of unmodified binding molecules that partition to the 'wrong' phase (V). Purification of the modified separator can, in such cases, give more efficient separation in the PALA procedure.

Such a purification can be performed with the same type of phase system as is used in the assay. However, a more efficient method may be preferred in this case, since a binding protein with a partition coefficient of approximately 1 will always be present in both phases and be difficult to separate off. The separation effect can in this case be enhanced by the use of a multi-step procedure, i.e. counter-current distribution. Another way to accomplish this is to shift to a chromatographic procedure. The partitioning in one phase system can be compared with one theoretical plate in a chromatographic column.

Some partitioning procedures have direct chromatographic counterparts, for example affinity partition with affinity chromatography and hydrophobic affinity partition with hydrophobic interaction chromatography. The basic partitioning procedure can be converted to partition chromatography (Martin and Synge, 1941), by adsorbing one phase in the phase system to the

stationary phase in the column and using the other as the mobile phase. Many examples of two-phase systems containing one organic phase can be found. If a broad definition of these techniques is used, most gas chromatography and high pressure liquid chromatography applications can be included in this context. Direct comparisons between the two methods have been made. For example, a hormone, oxytocin, which had been purified both with counter-current distribution using one aqueous phase and one organic phase was compared with partition chromatography using Sephadex G-25 and the same phases (Yamashiro, 1966).

Aqueous two-phase systems have also been used for the purification of DNA, with both CCD and partition chromatography methods. Here dextran was adsorbed on cellulose particles and PEG was used as the mobile phase (Müller et al, 1979).

Although partition chromatography using adsorbed dextran as the stationary phase has interesting possibilities, especially for basic research, the method has not been used very much. This may be because of practical reasons, the low long-term stability and the problem of efficient washing, resulting in the fact that the column may have to be loaded with a new phase system for every single run.

Separations utilising the weak hydrophobic effects that exist between two aqueous phases have interesting characteristics when compared to separations using conventional hydrophobic

interaction chromatography. In the latter version, alkyl- and phenyl-groups are used. Such groups are known to be able to penetrate proteins and may cause denaturation and decreased biological activity. Furthermore, the proteins may be strongly adsorbed to the ligands (Hofstee, 1979) and require chaotropic or other denaturing agents for elution (Påhlman et al, 1977). The risk of very strong adsorption also means that after a few runs, a hydrophobic column may become saturated with various immobilised peptides.

Aqueous solutions of poly-(ethylene glycol) are considered to be more hydrophobic than solutions of dextran or salts, as is shown schematically in the hydrophobic ladder (Fig. 3 in VI; Albertsson, 1971). Partitioning in aqueous two-phase systems has been ascribed to hydrophobicity when electrical and other effects have been cancelled (Zaslavsky et al, 1978; Albertsson, 1978). The use of these comparatively weak hydrophobic interactions should therefore be of interest in chromatographic systems, since they may offer a milder treatment for the chromatographed material.

Hydrophobicity is one of the variables in the expression for the partition coefficient (Albertsson, 1978) and can be used for analytical purposes. Measurements have been made of the partitioning of a homologous series of compounds having mixed hydrophilic and hydrophobic properties. These values have been used to construct a relative scale of hydrophobicity.

In paper VI is reported on the use of poly-(ethylene glycol), PEG, and poly-(vinyl alcohol), PVA, respectively, as "ligands", covalently linked to the matrix. The mobile phase consisted of a salt solution of rather high ionic strength. The covalent linkage of the ligands ensures physical stability and the column can be washed using the common procedures.

In the experiments with the PVA-gel, proteins were adsorbed at high ionic strength (1 - 3 M) and eluted at physiological ionic strength (0.15 M). Because no extra material could be washed off by using solutions containing etandiol or chaotropic salts, this is an indication that the proteins were not adsorbed as strongly as with conventional hydrophobic chromatography (VI).

PEG-modified glucose oxidase (VI), PEG-concanavalin A and PEG-antibody (unpublished), respectively, have been purified on PEG-Sephadex columns. For all three proteins, at least two peaks were obtained, one from the unretarded fraction containing native protein and one retarded containing PEG-modified protein. The retarded material always had a higher partition coefficient than the native protein, indicating that both the methods separated the material according to the same principles.

The experiments with bovine serum albumin on the PVA-gel (VI) show no retardation indicating that the protein is of hydrophilic nature. This is in contrast to similar experiments on alkyl-gels, whereby albumin is completely adsorbed at high

ionic strength (Hofstee, 1979). Albumin has hydrophobic sites which probably are responsible for the binding to alkyl groups. The fact that no adsorption is observed on the PVA-gel implies that no strong interaction with the sites occurs and that rather an overall hydrophobicity is measured. The "mild" hydrophobic chromatography may thus be regarded as a link between conventional hydrophobic chromatography and partitioning in aqueous two-phase systems.

CONCLUSION - PARTITION AFFINITY LIGAND ASSAY

The PALA method is intended to offer a convenient immunoassay procedure, based on the following properties:

- * The separation step is equipment independent. Only a test tube with the phase system is needed.
- * The whole assay - incubation, separation and measurement - can be carried out in one and the same vessel.
- * The same basic procedure can be used for the separation of low molecular weight compounds, proteins, as well as particles.
- * One of the reactants can be modified to give an extreme partitioning that is not sensitive to alterations in run conditions.
- * When an enzyme is used as a label, a semi-quantitative determination can be done by comparing the colour intensities in the two phases. The level where both phases are equally coloured may be adjusted to coincide with a critical concentration level.

The method of performing binding assays in aqueous two-phase systems can be utilized both for quantitative immunoassays, and for determinations of binding constants and the stoichiometry in binding studies.

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PARTITION AFFINITY LIGAND ASSAY (PALA). A NEW APPROACH TO BINDING ASSAYS

BO MATTIASSON and TORBJÖRN G.I. LING

*Department of Pure and Applied Biochemistry, Chemical Center, University of Lund,
P.O. Box 740, S-220 07 Lund, Sweden*

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A two-phase partitioning technique was used to separate free from bound labelled ligand in a binding assay. Efficient separation was accomplished by modifying one of the reactants in such a way that it partitions preferentially to one of the two phases. The biospecific interactions between the lectin concanavalin A and various carbohydrates and glycoproteins were investigated in this model study.

INTRODUCTION

One of the crucial steps in the use of routine binding assays (e.g., radio- and enzyme immunoassay) is the separation of free from bound reactant, and in recent years many approaches to overcome this problem have emerged. Since most available assay procedures require washing and centrifugation steps, these methods tend to be rather laborious and time-consuming.

The present paper deals with a new approach to a competitive binding assay based on separation, by partition, of biomolecules in aqueous two-phase systems. The two-phase systems used were formed by mixing solutions of two polymers or a polymer and a salt according to established procedures (Albertsson, 1971). Use of this technique in analysis requires an asymmetrical partition of free and bound molecules in the binding assay. In assays of haptens this may occur spontaneously (Mattiasson, 1980), whereas when assaying macromolecules this asymmetry in the system has to be created by modifying one of the reactants in the assay in such a way as to be partitioned preferentially in one of the phases, i.e., the two molecular entities are found in separate phases and the complex is recovered mainly from one of the phases.

MATERIALS AND METHODS

Concanavalin A was obtained from Miles-Yeda, Rehovot, Israel. Horseradish peroxidase, methyl- α -D-mannopyranoside, methyl- α -D-glucopyra-

noside and 4-amino-antipyrine were all obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Monomethoxypolyethylene glycol 2000 and 5000 (Carbowax) was a generous gift from Union Carbide, New York, NY, U.S.A. Polyethylene glycol 4000 and triazine were purchased from BDH, Poole, U.K. Dextran T-40 was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

PEG derivatization of concanavalin A

Monomethoxy-polyethylene glycol was activated according to previously published procedures (Abuchowski et al., 1976).

In a typical reaction 50 mg activated monomethoxy-polyethylene glycol (mol. wt. 5000) and 8 mg concanavalin A were mixed in 1 ml of a buffer consisting of 0.1 M triethanolamine-HCl, 1.0 M NaCl, pH 9.4. After 1 h at room temperature, the reaction was terminated by addition of 1.0 M glycine-NaOH, pH 9.4. Alternatively, the whole reaction mixture was dialysed overnight against the buffer used in the coupling step or left at 4°C overnight. Since the results obtained did not vary with procedure used, the reaction mixture was simply left at 4°C overnight before use.

Phase system used

There is a wide variety of potential phase systems. We chose one that contained no dextran and that separated fast after mixing, namely 13.5% (w/w) PEG-4000 and 13.5% (w/w) $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$.

Assay procedure

In a typical assay the carbohydrate and the labelled carbohydrate (in this case horseradish peroxidase was used as a naturally occurring glucoconjugate) were mixed prior to addition of the modified concanavalin A. The reaction volume was 100 μl . After equilibration at room temperature for a predetermined period, usually 10 or 30 min, the reaction mixture was partitioned in the two-phase system by addition of a well-mixed phase system consisting of 450 μl of each phase constituent. The tubes were vigorously shaken and the phases were allowed to separate for 10 min; 200 μl was then taken from each phase for determination of enzyme activity. The reaction mixture had a final composition of 100 mM Tris-HCl, pH 7.00, 14 mM phenol, 0.8 mM 4-amino-antipyrine and 1 mM hydrogen peroxide. The enzyme activity was determined at 510 nm with a spectrophotometer.

In an alternate assay procedure, the reaction mixture was mixed with the phase system and immediately poured into cuvettes and placed in a spectrophotometer (Zeiss PMQ II) in such a way that A_{510} was read in the bottom phase only.

Determination of K_{ass}

The assay was performed as described above with the only exception that

a predetermined amount of horseradish peroxidase together with a predetermined concentration of the carbohydrate to be tested was exposed to a fixed amount of modified concanavalin A. The degree of inhibition of peroxidase binding to the modified concanavalin A was taken as a measure of the binding affinity of the ligand.

RESULTS AND DISCUSSION

Analyses of mono- and polysaccharides, based on the binding of carbohydrates to concanavalin A, with the glycoprotein peroxidase from horseradish as labelled ligand are described here. Since concanavalin A binds to the carbohydrate portion of the enzyme, the latter can be regarded as a naturally occurring enzyme-carbohydrate conjugate (Borrebaeck and Mattiasson, 1980). To achieve an asymmetrical distribution of concanavalin A in the system, it was modified with monomethoxy-polyethylene glycol 5000 using triazine as a coupling agent. Partitioning constants K_{part} (i.e. the quotient of the concentrations in the top and bottom phases respectively) of more than 80 could be readily achieved compared with 0.031 for native concanavalin A.

When only peroxidase (2.5 nM) is present in the phase system the enzyme

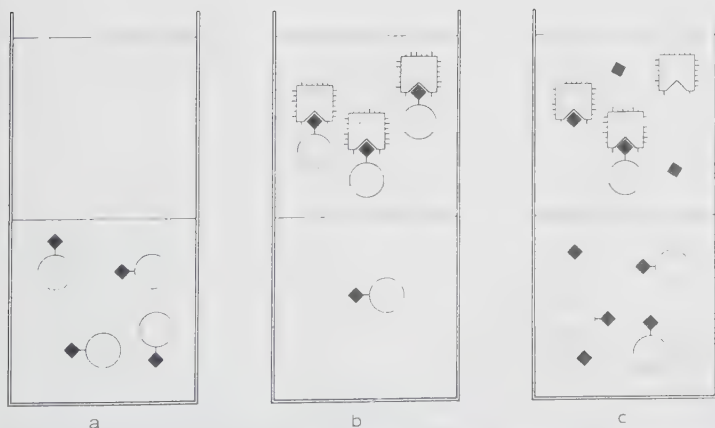


Fig. 1. Competitive binding assay based on the binding of the glucoenzyme peroxidase to the modified concanavalin A in an aqueous two-phase system containing 13.5% (w/w) polyethylene glycol (mol.wt 4000) and 13.5% (w/w) $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ and 10 mM sodium phosphate, pH 7.00. a: peroxidase (◆) (2.5 nM) is mainly found in the salt-rich bottom phase. b: modified concanavalin A (⌈) (1.8 μM) is in the polyethylene glycol-rich top phase as is the complex formed when peroxidase is bound to modified concanavalin A. The lectin thereby transports the enzyme to the top phase. c: when free carbohydrate (◆) that can bind to modified concanavalin A are added to the system they compete with free enzyme for the binding sites and the transportation is inhibited.

is found mainly in the bottom phase (Fig. 1a), with a partition coefficient of 0.063. If then modified concanavalin A is added to a final concentration of $1.8 \mu\text{M}$, the enzyme is transferred to the top phase (Fig. 1b) with a more than 40-fold increase in partition coefficient. Addition of glucose or mannose to the system competes with peroxidase for the binding sites on modified concanavalin A and thereby reduces the amount of peroxidase bound to the modified concanavalin A. The amount of enzyme found in the top phase is consequently diminished (Fig. 1c). The partition coefficient obtained for peroxidase at different concentrations of methyl- α -D-mannopyranoside in the phase system is shown in Fig. 2a.

In another set of experiments all of the reactants including the phase system and substrate for the enzyme-catalyzed reaction were mixed and the binding, partitioning and enzyme reaction were allowed to take place at the same time. Results from some experiments are shown in Fig. 2b which shows that this procedure can also be used successfully. Since the reproducibility of the time of contact between the reactants in a binding assay is essential under non-equilibrium conditions (Borrebaeck et al., 1978),

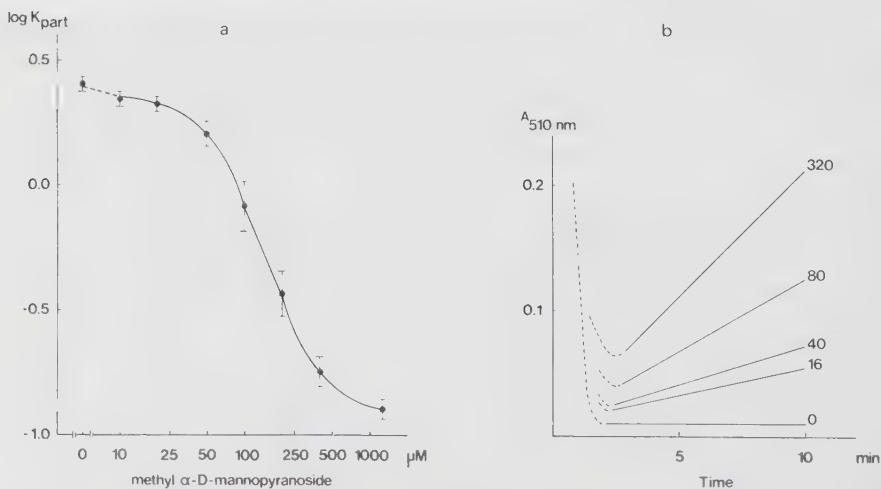


Fig. 2. a: calibration curve for methyl- α -D-mannopyranoside obtained for the system described in Fig. 1. Carbohydrate, enzyme and modified concanavalin A were incubated 10 min in a total volume of 100 μl . The two phase constituents were added to make a final volume of 1 ml and after mixing on a Vortex mixer the phases were allowed to separate for 10 min. Then 200 μl was taken from each phase for determination of enzyme activity. $\log K_{\text{part}}$ of the enzyme activity is plotted versus the logarithm of the carbohydrate concentration. b: time course for a system analogous to the one used in a, where all reactants, including phase components and substrates, were mixed. Immediately afterwards, the enzyme activity in the bottom phase was measured. The different concentrations of methyl- α -D-mannopyranoside are indicated on the graphs (in μM). The initial period of decreasing absorbance is due to phase separation.

it can be predicted that when a higher accuracy is needed a two-step procedure is to be preferred. This procedure, however, has the advantage of being carried out in one and the same vial.

The system studied here can be used for assaying concentrations of ligands in the μ molar range but it is not so sensitive as other binding assays based on antigen-antibody interactions (Mattiasson and Borrebaeck, 1980). It should be noted, however, that the K_{ass} for methyl- α -D-mannopyranoside to concanavalin A is $2.06 \times 10^4 \text{ M}^{-1}$ (So and Goldstein, 1968) as compared with the K_{ass} values for antigen-antibody interactions which are some orders of magnitude higher. The influence of the association constant for binding to concanavalin A of various carbohydrates on the sensitivity of the competitive binding assay was therefore examined. Equal concentrations of 4 different carbohydrates were tested and it was found (Fig. 3) that the sensitivity of the assay, reflected as $\log K_{part}$ in the partition step, is enhanced with a higher K_{ass} value. This observation is in good agreement with earlier findings using a lectin electrode (Borrebaeck and Mattiasson, 1980).

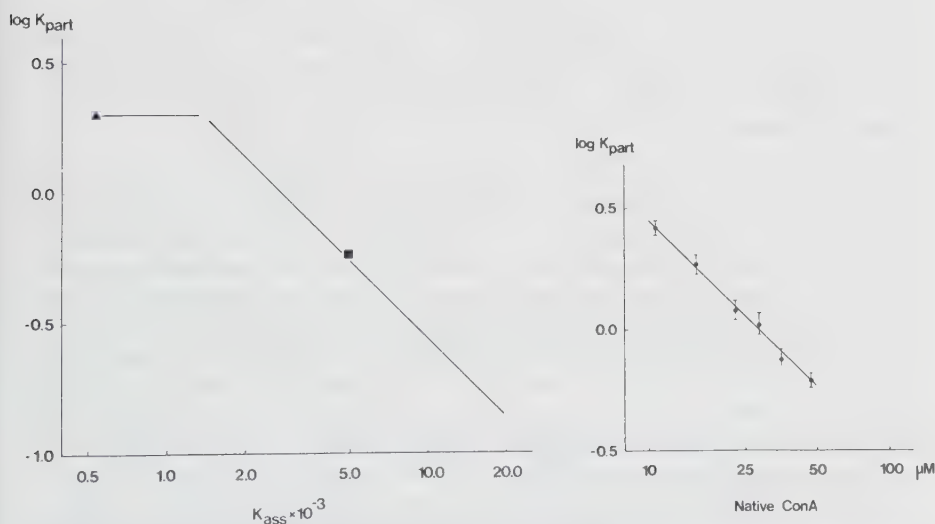


Fig. 3. The influence of association constants, $\log K_{ass}$, between free carbohydrate (3.3 mM) and concanavalin A on the sensitivity of the system shown in Fig. 1. Sensitivity is reflected by $\log K_{part}$ for peroxidase in the phase system. Symbols used: ▲, sucrose; △, D-fructose; ■, methyl- α -D-glucopyranoside; □, methyl- α -D-mannopyranoside.

Fig. 4. Standard curve for determination of native concanavalin A obtained by adding various amounts of native concanavalin A to the system described in Fig. 1b. Native and modified concanavalin A then compete for binding sites on peroxidase. $\log K_{part}$ for peroxidase is plotted versus the log of concentration of native Con A added.

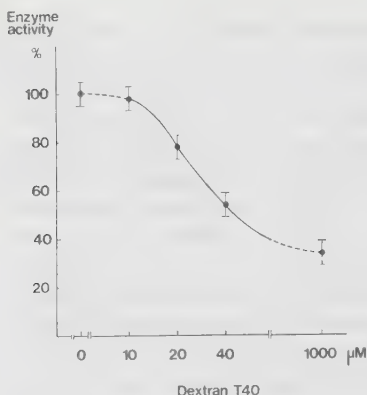


Fig. 5. Calibration curve for determination of a macromolecular carbohydrate, dextran T40, after competitive binding of dextran and peroxidase to modified concanavalin A. After partitioning had taken place, peroxidase activity in the top phase was assayed as a function of the dextran concentration. The enzyme activity scale is defined as: 0% = enzyme without modified concanavalin A added (see Fig. 1a); 100% = enzyme and modified concanavalin A but no dextran in the phase system (see Fig. 1b).

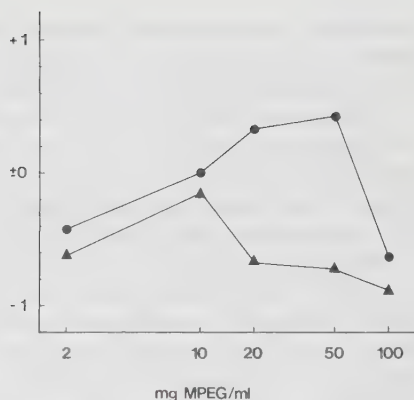


Fig. 6. The capacity of modified concanavalin A to transport peroxidase to the upper phase, depending on the degree of modification. Log K_{part} of the enzyme activity is plotted versus the logarithm of the amount of monomethoxy-polyethylene glycol used in the modification step. Symbols used: monomethoxy-polyethylene glycol mol. wt. 2000, ▲; mol. wt. 5000, ●.

The system described above was also used to investigate the possibility of determining the concentration of an unmodified binding protein. A calibration curve obtained after addition of various amounts of native concanavalin A to the system containing fixed amounts of modified concanavalin A and peroxidase is shown in Fig. 4. A prerequisite for the successful use of this variant of competitive binding assay is that the modified and unmodified protein have quite different partition coefficients. Since concanavalin A is present in 1000-fold molar excess over peroxidase, the partitioning of the enzyme depends on the proportion of the two forms of concanavalin A. The end points on the curve are situated in the proximity of the partitioning constants for modified and native concanavalin A, respectively.

As a model for assaying a macromolecular ligand, dextran (a polymer of glucose units), was quantified with the use of the concanavalin A-peroxidase system and a calibration curve is shown in Fig. 5.

The system described in this report was chosen mainly as a model to illustrate a new principle. This new technique involves some critical steps. The modification of the binding protein influences the binding properties to some extent. Fig. 6 shows a marked difference in lifting capacity of concanavalin A binding peroxidase when the degree of substitution was

increased. It should, however, be pointed out that Fig. 6 reflects an optimization between the binding properties and lifting properties. This question is receiving further attention. Another critical step when performing binding assays is the composition of the phases. Magnesium sulphate and polyethylene glycol were chosen here since the system is known to separate quickly (3–5 min). The most commonly used phase system in preparative phase-separation includes dextran and polyethylene glycol. Dextran can, however, not be used in this system since it interferes with concanavalin A. In other applications we have shown that many different phase systems can be used to achieve the expected phase separation. It should, however, be pointed out that though the phase system is not ideal, an analytical system capable of operating in the range of micromolar concentration could be achieved.

The method described in this paper can be compared with homogenous binding assays, e.g., enzyme multiplied technique (EMIT) (Rubinstein et al., 1978) and substrate-labelled fluorescence immunoassay (SLFIA) (Burd et al., 1977), in that no distinct separation or washing is necessary. The homogenous methods established so far have been applied only to low molecular substances even though development of assays for macromolecules have been discussed (Ullman, 1979). The partition affinity ligand assay (PALA), as illustrated in this report, may be used not only for small molecules, but also for macromolecules.

In conclusion, the use of modified binding molecules, 'separator molecules', in competitive binding assays in combination with phase partitioning offers a new possibility when designing assays based on biospecific interactions between ligands and receptors, e.g., antigens and antibodies, substrates and enzymes, carbohydrates and lectins, etc.

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APPLICATION OF PARTITION AFFINITY LIGAND ASSAY (PALA) IN A QUICK TEST FOR QUANTITATION OF *STAPHYLOCOCCUS* *AUREUS* BACTERIAL CELLS

BO MATTIASSON, TORBJÖRN G.I. LING and MATTS RAMSTORP

*Department of Pure and Applied Biochemistry, Chemical Center, University of Lund,
P.O. Box 740, S-220 07 Lund, Sweden*

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A novel immunochemical method for the quantitation of bacterial cells is described. The method, which is based on separation of bound from free reactants in aqueous two-phase systems has been studied both as direct and competitive binding assays, with a system consisting of intact cells of *Staphylococcus aureus* and human immunoglobulin G molecules.

To achieve high resolution, one of the reactants was modified so that the two reactants, the bacterial cells and the immunoglobulin molecules, occurred in different phases of the phase system. When binding takes place, the partition of one of the reactants is changed. The degree of change is then correlated to the amount of reactant present.

Using this method, cell numbers in the region 10^5 – 10^7 can be quantified. An assay takes 40–90 min.

INTRODUCTION

Quick diagnostic methods arouse great interest in microbiology, since successful treatment of a disease often depends on early recognition of the condition. Many of the typing procedures routinely used at present are, however, very time-consuming. Immunological techniques have proved both quick and sensitive and various approaches using specific immunological interactions in microbiological assays have been described (Danielsson and Kronvall, 1974; Hildebrand, 1979; Overby and Muskahwar, 1979).

This paper reports a binding assay for the quantitation of bacterial cells. The assay is based on interactions between surface structures on intact cells and specific binding molecules. In the system studied the interaction between protein A on the cell surface of *Staphylococcus aureus* strain Cowan I and human IgG molecules was used. The assay was performed in homogeneous solution and bound and free reactants were separated from each other using aqueous two-phase systems containing polymer/polymer or polymer/salt. These phase systems separate macromolecules, organelles and cells (Albertsson, 1971; Albertsson, 1978; Eriksson and Johansson, 1979) according to their individual surface properties.

Partitioning in aqueous two-phase systems was not used for competitive analysis of macromolecules before the introduction of heavily modified separator molecules (Mattiasson, 1980a). In a binding assay where two reactants are interacting, successful analysis requires that the two reactants are partitioned to different phases in the aqueous two-phase system. This situation may occur spontaneously, especially when assaying small molecules (Mattiasson and Ling, 1980), but otherwise one of the two reactants has to be modified so as to show a predilection for one or the other of the two phases, while the other reactant remains in the other phase. The analytical technique involving this combination of phase separation and competitive binding using separator molecules has been called Partition Affinity Ligand Assay (PALA) (Mattiasson, 1980a; Mattiasson and Ling, 1980).

The present report concerns the application of the PALA-principles in microbiological assays, here exemplified by the system *Staphylococcus aureus* and human immunoglobulin G, which besides being of interest per se may also be regarded as a model system for antigen-antibody reactions.

MATERIALS AND METHODS

Polyethylene glycols (PEG) of molecular weights 4000 and 6000 respectively, and triazine were obtained from British Drug House, Poole, U.K. Monomethoxypolyethylene glycol (M-PEG) 5000 (Carbowax) was a generous gift from Union Carbide, New York, U.S.A. Magnesium sulphate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, was from Merck, Darmstadt, F.R.G., Dextran T 250 from Pharmacia Fine Chemicals, Uppsala, Sweden, triethanolamine-HCl from Fluka AG, Buchs, Switzerland and immunoglobulin G (IgG) from KABI, Stockholm, Sweden. All other chemicals used were of analytical grade.

Heat-treated and formaldehyde-stabilized cells of *Staphylococcus aureus*, strain Cowan I (Kronvall and Frommel, 1970), was a generous gift from Pharmacia Diagnostics, Uppsala, Sweden.

Modification of the reactant with monomethoxypolyethylene glycol

Monomethoxypolyethylene glycol was activated with triazine according to Abuchowski et al. (1976). M-PEG-modified IgG was obtained by adding 20 mg activated M-PEG to a 1 ml solution of 8 mg IgG in 0.1 M triethanolamine-HCl, 1.0 M NaCl (pH 9.4). The mixture was agitated for 1 h at room temperature and finally stored at +4°C.

The *Staphylococcus* cells were modified by adding 2 mg activated M-PEG to 3×10^9 cells, in 1 ml 0.1 M triethanolamine-HCl, 1.0 M NaCl (pH 9.4). The cell suspension was kept under agitation at room temperature for 1 h and finally stored at +4°C.

Radiolabelling of human immunoglobulin G

Human immunoglobulin G was radiolabelled with 1 mCi of ^{125}I per mg protein by the lactoperoxidase method (Thorell and Johansson, 1971). The yield obtained was 91%.

Phase systems

Two different aqueous two-phase systems were used, containing polymer/polymer and polymer/salt, respectively. The polymer/polymer phase system was obtained by mixing 450 μ l 15% (w/w) Dextran T 250 and 450 μ l 10% (w/w) polyethylene glycol 6000, and the polymer/salt system was obtained by mixing 1 ml 30% (w/w) polyethylene glycol 4000 and 3 ml 30% (w/w) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.

Binding assays

Direct binding assay. In a direct binding assay of *Staphylococcus aureus*, a sample containing native bacterial cells and a predetermined number of PEG-derivatized ^{125}I -labelled IgG molecules was diluted to a total volume of 100 μ l by addition of 0.1 M phosphate buffer (pH 7.00). The mixture was stirred on a Vortex type mixer and left for 30 min at room temperature. The mixture was then subjected to partitioning in a polymer aqueous two-phase system obtained by mixing 450 μ l 10% (w/w) polyethylene glycol 6000 and 450 μ l 15% (w/w) Dextran T 250. The sample was mixed thoroughly and the phases were allowed to separate for 2 h at room temperature, after which a 200 μ l sample from the top phase was removed. Radioactivity was measured in a 1275 Minigamma, LKB Wallac (Bromma, Sweden) gamma counter.

Competitive binding assay. In a competitive binding assay of *Staphylococcus aureus* native bacterial cells compete with PEG-derivatized cells for a limiting number of ^{125}I -labelled IgG molecules.

A sample containing native *Staphylococcus* cells are mixed with a fixed number of PEG-derivatized cells and a predetermined number of ^{125}I -labelled IgG molecules. The total volume of the system of each sample was kept constant at 100 μ l by addition of 0.1 M phosphate buffer (pH 7.00). The mixture was thoroughly stirred on a Vortex type mixer and left for 30 min at room temperature. The mixture was then subjected to partitioning in a polymer aqueous two-phase system obtained as described in the case of the direct binding assay. A 200 μ l sample from the top phase was taken out for activity measurements in a gamma counter.

RESULTS AND DISCUSSION

The principle of using aqueous two-phase systems to separate bound from free reactant is schematically illustrated in Fig. 1. By modifying IgG with M-PEG the conjugate becomes more hydrophobic and shows a predilection for the PEG-rich top phase (Fig. 1a). When *Staphylococcus* cells are introduced into the system, IgG will be found in increasing amounts in the bottom phase since the IgG interacts with protein A on the cell surface and the cells are per se partitioned to the bottom phase (Fig. 1b).

The binding of a small amount of M-PEG-IgG molecules to the cells does not affect the partitioning of the cells in the system. If, however, there is a

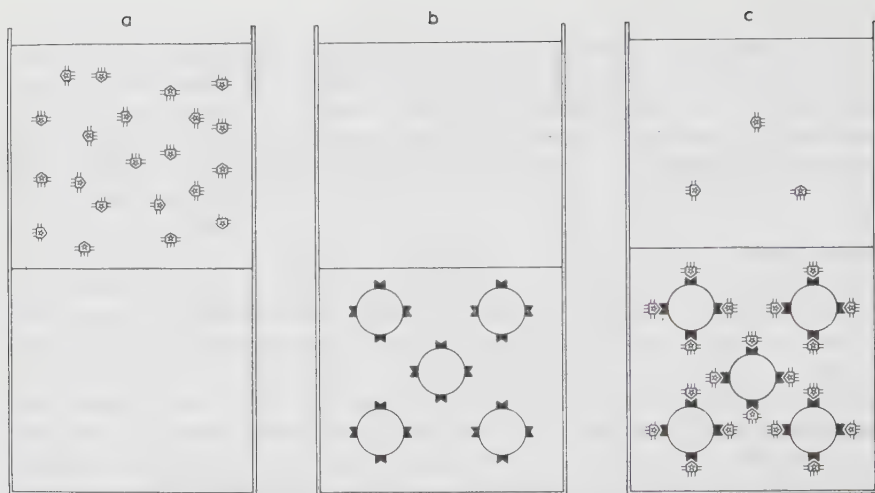


Fig. 1. Schematic representation of direct binding assay of *Staphylococcus aureus*. The cells are incubated with M-PEG-modified ^{125}I -labelled IgG and after 15 min bound IgG is separated from free IgG by partition in an aqueous two-phase system. The phase system consisted of PEG 4000 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mM sodium phosphate (pH 7.00). a: M-PEG-modified ^{125}I -labelled IgG partitioned mainly to the PEG-rich top phase. b and c: native *Staphylococcus aureus* cells partitioned mainly to the salt-rich bottom phase even in the presence of M-PEG-modified IgG and caused a drop in activity of the top phase.

large excess of M-PEG-IgG molecules in the system, the cells will be transported to the upper phase. The other extreme would be when there is an excess of binding positions on the cell surface so that virtually all M-PEG-IgG molecules will be bound and partitioned to the bottom phase. These two extreme situations limit the operational concentration range of the system.

An important step in the analytical procedure is the use of a proper degree of modification of the IgG molecule to make it partition to the PEG-rich phase without inhibiting the interaction with protein A on the cell surface of *Staphylococcus aureus*. To optimize the partition a series of M-PEG-IgG preparations with varying degrees of modification were prepared. Fig. 2 shows that a rather mild degree of derivatization gave an optimal effect. This optimal degree of derivatization was used in all subsequent experiments.

Fig. 3 gives a calibration curve from experiments with M-PEG-modified ^{125}I -labelled IgG molecules. Here the amount of radioactivity in the top phase is plotted as a function of the number of cells added to the reaction mixture.

It was expected from the competitive assay that an even more sensitive assay could be set up. However, a rough calculation of the counts available as iodine-labelled IgG for the assay suggested that a limiting factor in these

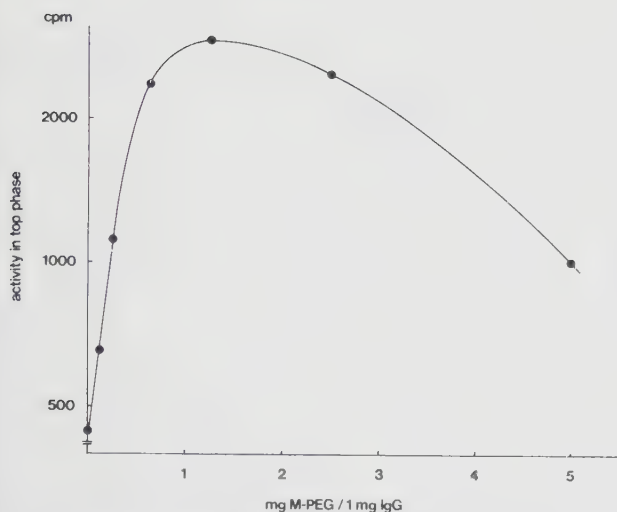


Fig. 2. Determination of the optimal degree of M-PEG-modification of IgG in a direct binding assay of *Staphylococcus aureus*. The diagram shows the activity in the top phase as a function of mg M-PEG added per mg IgG. The experimental conditions were: a predetermined number of *Staphylococcus aureus* cells were incubated for 30 min with a predetermined amount of ^{125}I -labelled IgG molecules modified with various amounts of M-PEG. The incubation mixture was allowed to partition for 30 min in an aqueous two-phase system as described in the legend to Fig. 1. A 400 μl sample was taken from the top phase for activity measurement.

assays is the rate of isotope disintegration. If a lower level of cpm in an assay is set at 200–300 in the phase analyzed, a theoretical lower level of detection may be calculated. The IgG preparation used in these experiments contained approximately 0.5 ^{125}I per IgG molecule. The rate of disintegration of the radioactive isotope is such that in each assay approximately 10^{11} ^{125}I must be present to give 200 cpm. Since the surface of each *Staphylococcus aureus* cell has approximately 10^6 binding sites for IgG (Kronvall and Frommel, 1970), a lower limit will lie in the region of 10^5 cells. The use of more highly labelled IgG may improve the sensitivity of the assay, but only marginally.

It has been argued that for several reasons enzyme labels are preferable to radioactive isotopes (Wisdom, 1976). Such arguments have been the medical hazard involved in the handling of the radioactivity, the short shelf-life of the labelled reagents and the risk that gamma-radiation may destroy vital structures of the labelled molecule (Wisdom, 1976; Mattiasson and Borrebaeck, 1980). Still another argument can now be added, namely the limiting sensitivity. If enzyme labels are used, even small numbers of labelled molecules can be quantified and, from a theoretical point of view,

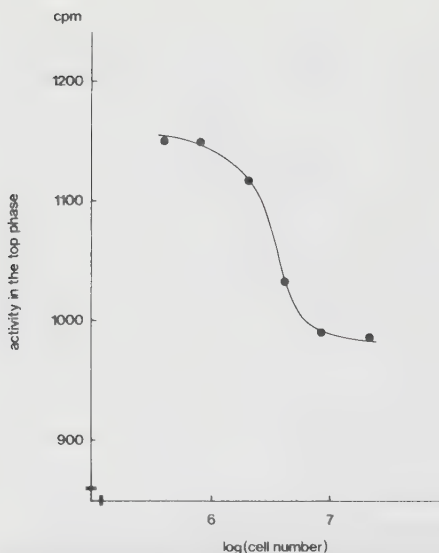


Fig. 3. Calibration curve for *Staphylococcus aureus* obtained in a direct binding assay. The diagram shows the activity in the top phase as a function of the number of *Staphylococcus aureus* cells present in the sample. A fixed amount, 1.2×10^{-13} mol of M-PEG-derivatized ^{125}I -labelled IgG molecules were incubated for 30 min with various numbers of native *Staphylococcus aureus* cells. The incubation mixture was then partitioned for 30 min in an aqueous two-phase system as described in the legend to Fig. 1. A 200 μl sample was taken from the top phase for activity measurement.

the sensitivity of the assay is improved. Preliminary studies using enzyme-labelled reagents show that a higher sensitivity of such an assay is possible.

When using M-PEG-modified IgG molecules as separators, the operating concentration range expressed as the number of cells per ml that can be measured is governed by the intricate balance between too many or too few binding sites. If the number of cells is too small, all binding sites will be occupied and thus no resolution can be expected. On the other hand, if the number of separator molecules bound per cell is increased, the affinity for the bottom phase of the cell will be counteracted by the affinity for the top phase of the modified IgG, with the result that the cell-IgG-M-PEG complex will stay in the interface or, when high binding has taken place, the cells will stay in the top phase. At the other extreme where such an analysis is applicable, an excess of cells will tend to bind all IgG-M-PEG molecules and no differentiation between the different cell counts can be expected.

The two different approaches to bacterial cell quantitation presented here will operate well within concentrations down to 10^6 cells per ml. However, owing to the potential complications with enrichment of cells at the interface, the competitive assay approach may be preferred.

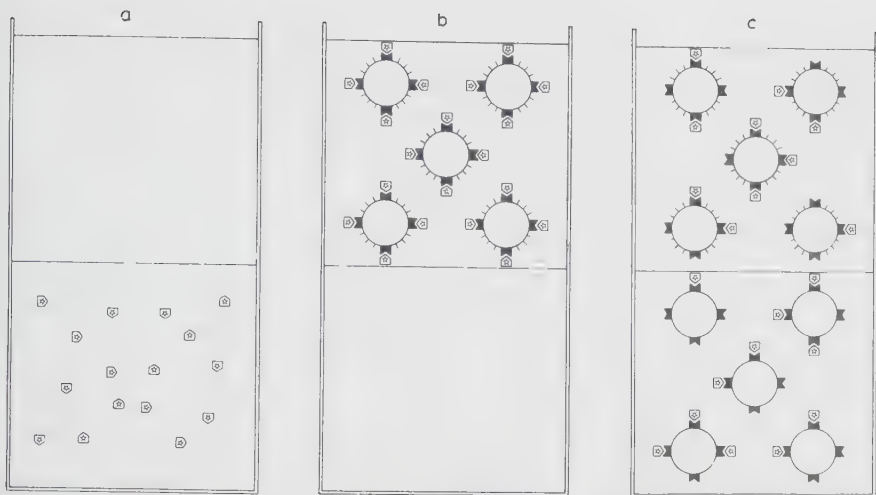


Fig. 4. Schematic representation of a competitive binding assay of *Staphylococcus aureus* where a limited number of ^{125}I -labelled IgG molecules binds to native and M-PEG-modified *Staphylococcus aureus* cells respectively. a: ^{125}I -labelled IgG partitioned mainly to the salt-rich bottom phase. b: M-PEG-modified *Staphylococcus aureus* cells partitioned mainly to the PEG-rich top phase. IgG bound to the cells were thereby transported to the top phase. c: Native *Staphylococcus aureus* cells partitioned mainly to the bottom phase. Since competition between native and M-PEG-derivatized bacterial cells for ^{125}I -labelled IgG takes place in the incubation mixture, a shift in the distribution of ^{125}I -labelled IgG occurs on addition of native cells.

In earlier studies (Mattiasson, 1980a, b; Mattiasson and Ling, 1980), when soluble reactants were used, separation was quick and sensitivity high. This could also be achieved in cell analysis provided whole cells are used as transporters of small marker molecules across the interface.

Most work on immunological analysis in microbiological systems has so far dealt with soluble antigens from the microorganism. It would be easier to analyse such a system, than a system containing whole cells, using the PALA technique. There is no reason to doubt the possibility of a mixed analysis of cells plus free antigens.

In the competitive assay studied, a fixed number of M-PEG-derivatized cells of *Staphylococcus aureus* are competing with various numbers of native cells for a limited number of ^{125}I -labelled IgG molecules. The principle of the competitive binding assay using the PALA method is shown in Fig. 4. To assess the capacity of PEG-modified cells of *Staphylococcus aureus* to transport ^{125}I -labelled IgG molecules to the other side of the interface, a simple titration test was performed. Varying amounts of ^{125}I -labelled IgG molecules were added to a constant amount of M-PEG modified *Staphylococcus aureus* cells. After binding followed by separation in the phase

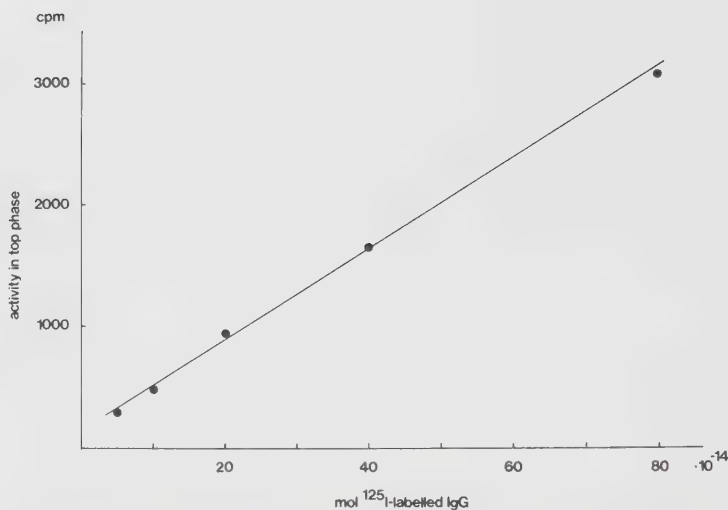


Fig. 5. Titration curve obtained by direct titration of a constant number 6×10^7 of M-PEG-derivatized *Staphylococcus aureus* cells with ^{125}I -labelled IgG. The diagram shows the activity in the top phase obtained when increasing amounts of ^{125}I -labelled IgG were incubated with the cells. After 30 min incubation the mixture was allowed to separate in an aqueous two-phase system consisting of PEG 4000 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mM sodium phosphate (pH 7.00).

system, a sample was taken from the top phase for analysis in a gamma counter. The result of such a series of experiments is shown in Fig. 5. A linear relationship was found between the amount of labelled IgG bound to the cells, measured as counts transported across the interface, and the amount of ^{125}I -labelled IgG added. Thus, the amount of M-PEG-modified cells is sufficient for the amount of labelled IgG added, since no signs of saturation appeared under the experimental conditions used. These conditions were then used in a competitive assay between modified and native *Staphylococcus aureus* cells for ^{125}I -labelled IgG. The results obtained are shown in Fig. 6. As seen from this figure, cells in the region of 10^5 – 10^7 were measured.

The results clearly show that the PALA-technique can accelerate and facilitate microbiological assays. The time needed for phase separation to take place depends largely on the phase constituents used. When PEG/Dextran is used it takes approximately 1 h, whereas when PEG/ MgSO_4 is used separation is complete within 10 min. This makes the total time for one assay 40–90 min. However, still much work remains to be done before a useful method can be developed and applied in practice.

It should be pointed out that the system studied is a model system, and

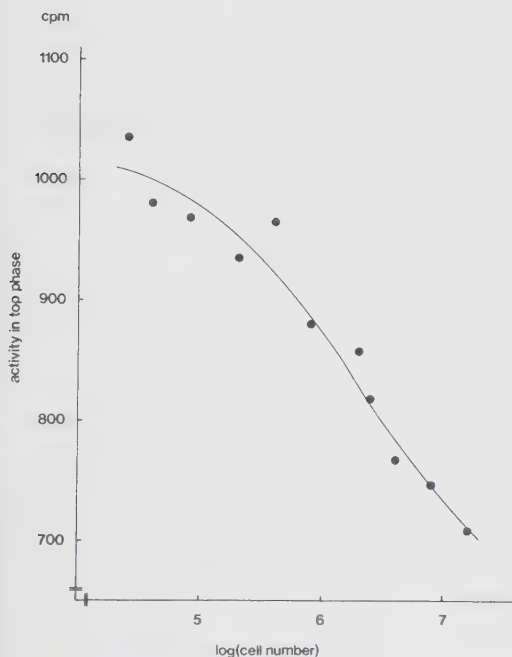


Fig. 6. Calibration curve obtained for *Staphylococcus aureus* cells obtained in a competitive binding assay, using a constant amount of ^{125}I -labelled IgG molecules, 2.6×10^{-14} mol. The diagram shows activity in the top phase as a function of number of native *Staphylococcus aureus* cells added to 6.3×10^5 M-PEG-modified *Staphylococcus aureus* cells. The mixture was incubated with ^{125}I -labelled IgG for 30 min and thereafter allowed to separate in an aqueous two-phase system consisting of PEG 6000 and Dextran T 250. A 200 μl sample was taken from the top phase for measurement of activity.

that we have taken advantage of the fact that protein A on the *Staphylococcus aureus* cell surface specifically binds IgG molecules. Work is now in progress with other systems involving antigen-antibody interactions as well as use of enzyme-labelled reagents.

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Immunological Quantitation of Bacterial Cells Using a Partition Affinity Ligand Assay: A Model Study on the Quantitation of Streptococci B

T. G. I. LING, M. RAMSTORP, AND B. MATTIASSEN

*Department of Pure and Applied Biochemistry, Chemical Center, University of Lund,
P.O. Box 740, S-220 07 Lund, Sweden*

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A new immunoassay for bacterial cells is described. Aqueous two-phase systems were used to separate the different reactants after binding, the principle being that in the direct assay free and bound reactants shall be recovered from different phases. In the competitive binding assay the bacteria are present in two forms—one native (to be quantified) and one modified (in fixed amount)—so that after binding and separation the complexes are recovered from different phases. ^{125}I -Labeled antibodies were used in the competitive assay and enzyme-labeled antibodies in the direct binding assay. In the system studied here streptococci were quantified. The direct enzyme immunoassay was more sensitive than the competitive radioimmunoassay. The smallest number of cells that could be quantified was 2500. Total time for one assay was 40–120 min.

In recent years immunochemical methods have proved useful in the analysis of bacteria. Many new immunological assays have been developed using the concepts of radio- and enzyme-immunoassay (1). Most of these assays detect low-molecular-weight antigens. The development of immunoassays for intact bacteria has, however, been impeded by difficulties in separating free from bound reactants, since such assays require a separation method that can handle both whole cells and proteins.

Partitioning in aqueous two-phase systems has recently proved useful in the separation step in binding assays of low-molecular-weight ligands and macromolecules (2,3) as well as of whole cells (4). This method has been called partition affinity ligand assay (PALA). In an assay of cells, free and bound labeled antibodies are separated in a two-phase system after the binding step, and the amount of label in one of the phases is determined. A prerequisite for an immunoassay of cells in a two-phase system is that the cells to be quantified and free antibodies are partitioned differently so that antibodies not

bound by the cells to be quantified are found in one phase and the bound ones in the other. In some cases it was possible to design a phase system in which the free and bound labeled antibodies partitioned spontaneously to different phases. A more general approach was to modify one of the reactants in order to make virtually all of it partition to one of the phases and thereby improve the separation considerably. The modification was effected by covalent attachment of one of the polymers used in the phase system.

Streptococci of type B1 were chosen for this study as they are ubiquitous and responsible for infections with a wide variety of clinical manifestations (5). There is thus a need for quick and reliable analytical methods, especially in cases where an infection may become a serious complication and even threaten the life of the patient (6).

MATERIALS AND METHODS

Materials. QAE-Sephadex A-50, Sephadex G-200, PD-10 columns (containing Sephadex G-25), and Dextran T250

were obtained from Pharmacia Fine Chemicals AB, Uppsala. Triazine and poly(ethylene glycol) with a molecular weight of 6000 came from British Drug House, Poole. Monomethoxy-poly(ethylene glycol) (MPEG,¹ trade name Carbowax) with a molecular weight of 5000 was a generous gift from Union Carbide, New York. Triethanolamine-HCl was obtained from Fluka AG, Buchs, and 4-aminoantipyrine and horseradish peroxidase (type VI) from Sigma Chemical Company, St. Louis, Missouri. Antiserum against *Streptococcus* B1 came from BBL, Division of Becton, Dickinson and Company, Cockeysville, Maryland, and from Lee Laboratories, Inc., Grayson, Georgia. All other chemicals used were of analytical grade.

Streptococcus B1 cells, heat treated and stabilized with formaldehyde, were a generous gift from Pharmacia Diagnostics AB, Uppsala. Cells were counted in a Bürker chamber using a phase-contrast microscope ($\times 400$). Attempts were made to count individual cells.

Modification of streptococci with monomethoxy-poly(ethylene glycol). MPEG was activated with triazine (7). In the coupling step activated MPEG was added to a suspension of streptococci (1.2×10^8 cells/ml) with 75 mmol/liter triethanolamine-HCl, pH 9.4. Unless otherwise stated, 10 mg activated MPEG was added per milliliter of suspension. After agitation for 1 h at room temperature the cells were stored at 4°C.

Purification of antibodies. Antibodies were purified from whole serum by ion-exchange chromatography on a QAE-Sephadex A-50 column equilibrated with sodium phosphate buffer, pH 7.00, ionic strength 0.10 mol/liter. The fraction that was not adsorbed to the ion exchanger was collected and concentrated.

¹²⁵I-labeled antibodies. Antibodies were prepared with the lactoperoxidase method

(8). One millicurie of ¹²⁵I was added to 0.85 mg of antibodies in 0.04 mol/liter sodium phosphate buffer at pH 7.00 and incubated for 10 s at room temperature. Free ¹²⁵I was separated from the antibodies in a PD-10 column. The yield in the labeling step was 90%.

Enzyme-labeled antibodies. Antibodies were prepared with the sodium meta-periodate method (9). One milligram of horseradish peroxidase was activated and mixed with 2 mg antibody. The conjugate formed was separated from unreacted protein on a Sephadex G-200 column (1.6×72 cm).

Competitive binding assay. In the competitive binding assay streptococci in a sample were mixed with a fixed amount of MPEG-modified streptococci and allowed to compete for a limited amount of ¹²⁵I-labeled antibody. The total volume was 100 μ l and contained 0.1 mol/liter sodium phosphate buffer, pH 7.00. The reagents were mixed and incubated at room temperature for 30 or 60 min and then separated as described below.

Direct binding assay. In the direct binding assay streptococci in a sample were mixed with a fixed amount of antibody labeled with peroxidase in a total volume of 100 μ l. The buffer used consisted of 0.010 mol/liter Tris-HCl, 0.090 mol/liter KCl, pH 7.00. The buffer and the phase system components were autoclaved before use. After partitioning as described below a 200- μ l sample was taken from the top phase and mixed with 800 μ l reagent solution, giving a final concentration of 14 mmol/liter phenol, 0.8 mmol/liter 4-aminoantipyrine, 1 mmol/liter hydrogen peroxide, and 10 mmol/liter Tris-HCl, pH 7.00. The enzyme activity was measured in a spectrophotometer at 510 nm.

Separation using a two-phase system. In a typical assay 900 μ l of a premixed phase system consisting of 5% (w/w) PEG-6000 and 7.5% (w/w) Dextran T250 was added to 100 μ l of incubation mixture and thoroughly mixed on a Vortex-type mixer. The mixture was left for 60 min at room tem-

¹ Abbreviation used: MPEG, monomethoxy-poly(ethylene glycol).

perature to allow the phase separation to take place. Two distinct phases were formed and the free and bound antibodies were separated to different phases. A 200- μ l aliquot was taken from the top phase (or from each phase) and the amount of labeled antibody was determined. The distribution of antibodies is expressed by the partition coefficient, defined as the ratio between the concentrations in the top and bottom phases, respectively: $K_{\text{part}} = C_{\text{top}}/C_{\text{bottom}}$.

RESULTS AND DISCUSSION

Bacterial cells were quantified with both competitive and direct binding assays. The latter assay is simpler but requires that the cells and the antibodies partition to different phases. However, when studying the spontaneous partitioning of the native reactants in an aqueous two-phase system, it was found in the case of streptococci that the cells and the antibodies both partition to the bottom phase and that no effective separation of the reactants could be achieved, not even when the ionic composition of the phase system was varied. In such a situation it is possible to change the partitioning by modification of the surface properties of either of the two reactants in the binding assay (3,4). The species (here streptococci B cells) to be quantified is for practical reasons not suitable for chemical treatment. Furthermore, modification of the antibodies may lead to deactivation. A search was therefore made for other solutions.

In the competitive binding assay (Fig. 1), varying amounts of streptococci from a sample and a fixed amount of MPEG-modified streptococci are incubated with a limiting amount of labeled antibody. The incubation mixture is then partitioned in an aqueous two-phase system. The modification aims at getting the two bacterial reactants to partition to different phases in the separation step. In the phase system used, consisting of PEG and Dextran, unmodified streptococci partition mainly to the Dextran-rich bottom

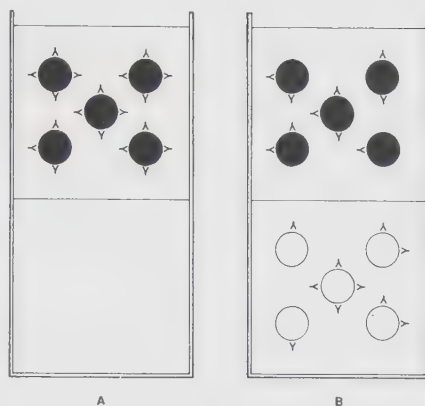


FIG. 1. Schematic representation of the competitive assay. Unmodified bacteria from the sample and PEG-modified bacteria are mixed with ^{125}I -labeled antibodies and incubation for 60 min. The phase system is added, and after 60 min for separation the radioactivity in one or both of the phases is determined. (A) MPEG-modified streptococci (●) partition to the top phase and thereby transport bound antibodies (Y). (B) When unmodified streptococci (○) are present they compete with the MPEG-modified streptococci for the antibodies. Since the unmodified cells partition to the bottom phase the result is a decrease in the activity in the top phase.

phase. The modified cells should then be found in the PEG-rich top phase, so that a competitive situation can be exploited in the binding step and, in the subsequent partitioning step, the distribution of the bound labeled antibody will reflect the relative amounts of unmodified and modified cells, respectively.

In an assay for *Staphylococcus aureus* (4), the cells were made to partition almost exclusively to the top phase by covalent attachment of MPEG. It was found that there is an optimum degree of modification achievable by variation of the amount of MPEG molecules added per cell or biomolecule. At a low degree of modification the surface properties are not changed enough to bring about satisfactory partitioning. On the other hand, when too many MPEG molecules are attached they may be a steric hindrance to the binding of antibodies to the antigenic determinants. For the streptococci an opti-

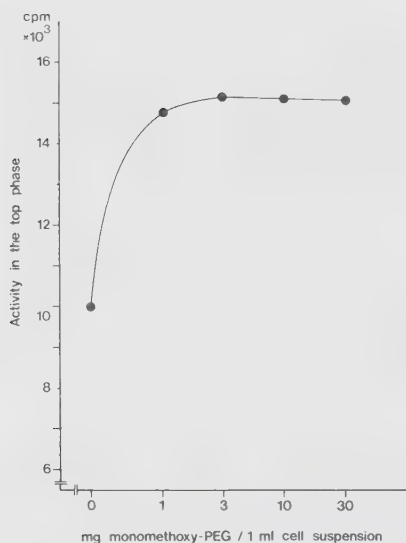


FIG. 2. Determination of the capacity of MPEG-modified streptococci to transport antibodies to the top phase for different degrees of modification using the conditions described for the competitive assay. The activity in the top phase is shown as a function of the amount of activated MPEG added per milliliter of reaction mixture (1.2×10^8 cells/ml).

imum degree of MPEG modification was obtained with 3 mg activated MPEG added per 10^8 cells, as determined by the partitioning of bound ^{125}I -labeled antibodies (Fig. 2). The partitioning was not significantly altered by a 10-fold larger amount of MPEG, which allowed a high degree of modification without significant loss of transport capacity under the conditions used. In all subsequent experiments a modification of 10 mg MPEG per 10^8 cells was used. Under the experimental conditions used, the partitioning of ^{125}I -labeled antibodies bound to the modified streptococci did not vary with the amount of antibody added; i.e., the bound antibodies did not markedly affect the partitioning of the cells. The amount of labeled antibody found in a phase was therefore taken as a function of the number of cells in that phase.

When designing a competitive binding

assay it is important to select the proper amounts of reagents that are to be used in the assay. If a fixed amount of ^{125}I -labeled antibodies is used the number of MPEG-modified cells necessary to transport the antibodies to the top phase has to be determined (Fig. 3).

The conditions for the partitioning were chosen in such a way that unmodified streptococci partitioned to the bottom phase. The partitioning of the PEG-modified cells was rather insensitive to variation of the composition of the phase system. It was thus possible to optimize the partitioning so that one of the two bacterial entities partitioned completely to one phase, and the other to the other phase. The partitioning of the labeled antibodies was, however, not critical since the sites on the bacterial cells were present in heavy excess. The specific antibodies could therefore be assumed to be bound to the cells. Thus, the partitioning of the label is a function of the ratio between the number of modified and the number of unmodified

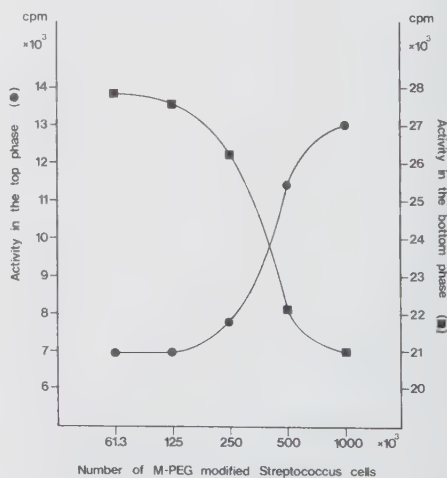


FIG. 3. Determination of the proper amount of MPEG-modified bacteria needed to transport antibodies to the top phase. The degree of modification used was 10 mg activated MPEG/ml. The conditions described for the competitive assay were used.

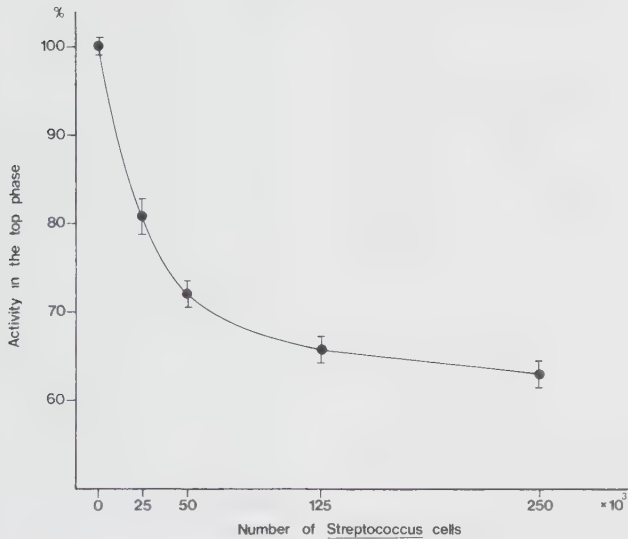


FIG. 4. Standard curve for the competitive assay of streptococci under the experimental conditions described in the text. The number of MPEG-modified streptococci present was 30,000. On the activity scale 0% denotes the activity with no cells in the system and 100% the activity with only MPEG-modified cells present.

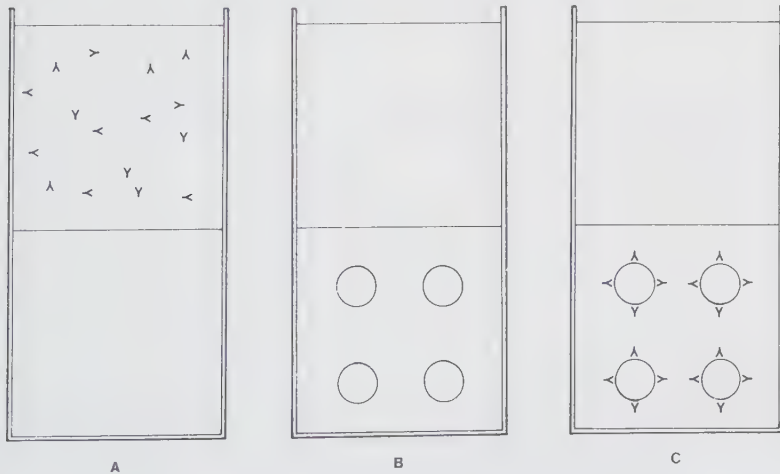


FIG. 5. Schematic representation of the direct binding assay. Unmodified bacteria from the sample and enzyme-labeled antibodies are mixed and incubated for 60 min. The phase system is added and after another 60 min for separation the enzymatic activity in the top phase is determined. (A) The enzyme antibody conjugate (Y) partitions mainly to the top phase. (B) The unmodified streptococci (O) partition to the bottom phase. (C) When the enzyme-labeled antibodies are bound to the bacteria, they are transported to the bottom phase with a consequent decrease in the enzyme activity of the top phase.

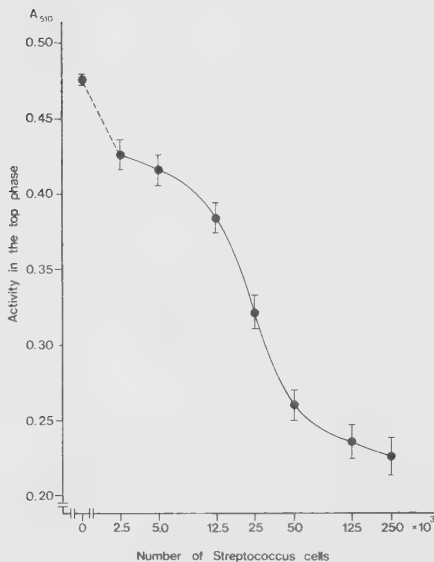


FIG. 6. Standard curve for the direct binding assay of streptococci using the experimental conditions described in the text. A sample from the top phase is drawn and added to a solution containing substrates for the enzyme. The absorbance is read after a predetermined time, usually 1 h.

bacteria, respectively. A standard curve for the quantitation of streptococci was obtained (Fig. 4).

The whole immunoglobulin G fraction of the antiserum was labeled and used in the assay, with the result that there was a large amount of antibody without specificity for the streptococci present. Consequently, there was a high background activity (Fig. 3), which limited the sensitivity of the assay. This limitation can, if necessary, be overcome by further purification of the antibodies with, e.g., affinity chromatography. Under the experimental conditions used, the sensitivity of this competitive assay depends mainly on the competitive situation *per se*, since the smallest detectable amount of streptococci in the assay cannot be much smaller than that of the MPEG-modified cells used.

The limiting factors of a competitive sys-

tem can be obviated by the use of a direct binding assay in which the binding of antibodies to cells is measured (Fig. 5). A prerequisite is that the two reactants partition differently in a two-phase system so that the bacteria can transport bound antibodies across the phase boundary. In the direct binding assay illustrated in Fig. 5, the antibodies were labeled with an enzyme, giving an enzyme-antibody conjugate with an appropriate partition coefficient so that further modification was unnecessary. The partitioning of the reactants was optimized by replacing the sodium phosphate buffer used in the competitive assay with a buffer consisting of Tris/KCl. The standard curve for the direct assay is shown in Fig. 6, from which it is clear that the sensitivity was significantly better than that of the competitive assay.

The partitioning of molecules or cells in a two-phase system is not sensitive for other molecules or cells present in the system. The separation is thus not influenced by, e.g., serum or cell homogenate, as long as the ionic strength and pH of the system are not changed. The PALA procedure has been used for determination of triiodothyronine in serum with about the same precision and accuracy as obtained with commercial kits (10).

A complication in partitioning cells is the possibility of adsorption of cells to the interface. Cells may be distributed between one of the two bulk phases and the interface (11), but the adsorption can often be kept at a low level by the use of a suitable phase system. With the PEG-Dextran system used, it was found that on addition of MPEG-modified streptococci the decrease in activity in one phase was balanced by an increase in the other (Fig. 3), indicating that the adsorption to the interface did not affect the outcome of the assay.

When quantifying cells with immunological procedures not only viable cells are detected, but also dead cells and cell fragments with the antigenic determinant. Therefore,

an immunoassay may give a higher number of cells than what is obtained with counting procedures. In a study where yeast cells (*Saccharomyces cerevisiae*) were quantified by the binding of radiolabeled concanavalin A to the cell surface (12), it was found that the PALA procedure gave about 5% higher values than those obtained by counting in a Bürker chamber.

Identification of streptococci has conventionally been based on the extraction of group-specific surface carbohydrate antigens (13) and subsequent identification with immunological methods. In order to compensate for the low sensitivity in immunoprecipitation methods the bacteria must be cultured overnight before analysis (14). Recently, a more sensitive immunoassay has been applied in the grouping of whole streptococci (15), but the cells must still be cultured overnight.

The present method is quick (40–120 min) and sensitive. In the development of new analytical techniques for bacterial cells it is desirable to try to design assays sensitive enough to make preculture of the cells unnecessary.

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COMPARISON BETWEEN BINDING ANALYSES PERFORMED BY EQUILIBRIUM DIALYSIS AND PARTITIONING IN AQUEOUS TWO-PHASE SYSTEMS EXEMPLIFIED BY THE BINDING OF CIBACRON BLUE TO SERUM ALBUMIN

T. G. I. LING* and B. MATTIASSON

Department of Pure and Applied Biochemistry, Chemical Centre, P.O. Box 740, S-220 07 Lund (Sweden)

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SUMMARY

Partitioning in two-phase systems was used as a separation method in binding analyses and compared with equilibrium dialysis. A phase system was chosen to give partitioning conditions with the reactants in different phases. The interaction between serum albumin and Cibacron Blue F3G-A was used as a model system. The protein partitioned to the bottom phase and the ligand to the top phase in a phase system consisting of poly(ethylene glycol) and dextran. When both albumin and Cibacron Blue were present in the phase system, the degree of binding was observed as a translocation of ligand from the top to the bottom phase. An evaluation method that compensates for the amount of free reactant in the phase with the binding protein is presented.

INTRODUCTION

In binding assays there are two principal ways to determine the degree of binding between two reactants. If the complex formed has a property that is unique in relation to those of the participating single reactants present, this property can be used for measurement in a homogeneous solution. Otherwise, bound and free reactants must be separated in order to perform the measurement. In the second alternative, the speed and efficiency of the separation greatly influence the outcome of the experiment. If the separation is very effective, it may lead to a dissociation of the complex formed. When, *e.g.*, the binding of a ligand to a protein is studied, removal of the free ligand will displace the state of equilibrium. Further, this reaction is time dependent, which makes the time allotted for the separation procedure important¹. These factors can evidently affect the values obtained in a binding analysis. However, they are difficult to determine and are rarely discussed in the literature. They may nevertheless explain discrepancies between different methods using different separation procedures.

In this work, partition in aqueous two-phase systems was used for the separation. This technique is simple and fast and has proved very useful in im-

munoassays²⁻⁵. The points mentioned above of importance for the performance of an assay can be kept under control by choosing a phase system that gives adequate difference in partitioning of the bound and the free reactant. Partitioning has also been used in binding analyses^{6,7}, but up to now only with the approximation that the binding protein partitions exclusively to one of the phases. Separation in two-phase systems is not only useful when interactions between a binding protein and a ligand are studied, but also between different macromolecules² and between cells and proteins^{3,4}. The binding reaction between albumin and the dye Cibacron Blue⁸ is used as an example to demonstrate how a system is set up and how one evaluates the experimental results when the reactants do not completely partition to different phases.

MATERIALS AND METHODS

Human serum albumin, "essentially free of fatty acids", was purchased from Sigma (St. Louis, MO, U.S.A.). Cibacron Blue F3GA was purchased as Reactive Blue from Sigma and deactivated by reaction with glucose⁹. All other chemicals used were of analytical-reagent grade.

Equilibrium dialysis

Equilibrium dialysis was performed in cylindrical cells (40 × 25 mm I.D.) made of Delrin, divided by a circular dialysis membrane ("seamless cellulose tubing"; Union Carbide, New York, NY, U.S.A.).

Partitioning

Albumin and the dye were incubated for 10 min in a volume of 100 μ l, then 900 μ l of a well mixed phase system was added to make a final concentration of 0.070 g/g of PEG 6000 (Union Carbide) and 0.100 g/g of Dextran T-70 (Pharmacia, Uppsala, Sweden). Alternatively, a phase system with a final concentration of 0.135 g/g of PEG 4000 and 0.135 g/g of magnesium sulphate heptahydrate was used. After separation (30 min), an aliquot of 200 μ l was taken from each phase and diluted to 1 ml with buffer and the amount of dye was measured spectrophotometrically at 610 nm. An absorption coefficient of $1.04 \cdot 10^4$ l mol⁻¹ cm⁻¹ was used in all calculations.

Kjeldahl analysis

Kjeldahl analysis of protein content was carried out using a Kjeltec System II from Tecator AB (Helsingborg, Sweden).

THEORETICAL

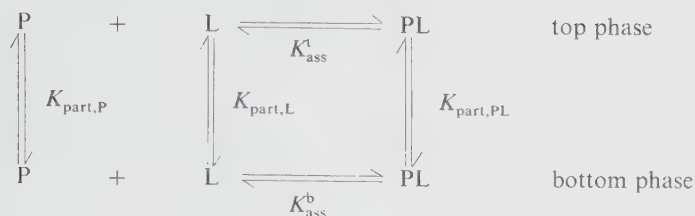
The partitioning of a molecule in a phase system is characterized by the partitioning coefficient, K_{part} , defined as

$$K_{\text{part}} = C_{\text{top}}/C_{\text{bottom}} \quad (1)$$

If a ligand, L, is present in a two-phase system together with a binding protein, P, the following scheme shows the situation, where superscript t denotes the top phase and b

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the bottom phase



A prerequisite for a binding assay in aqueous two-phase systems is that the reactants favour different phases, so that the degree of binding is observed as a displacement of the observed reactant, in this instance the ligand. The stoichiometry of the binding reaction is not considered at this point. In the binding analysis, the amount of ligand present in each phase after separation is determined. In each phase the ligand is present both free and bound to the binding protein. This can be expressed as in the following equations, with the symbols used here:

$$C_{\text{L,tot}}^t = C_{\text{L}}^t + C_{\text{PL}}^t \quad (2)$$

$$C_{\text{L,tot}}^b = C_{\text{L}}^b + C_{\text{PL}}^b \quad (3)$$

The total amount of complex formed, irrespective of the partitioning, has to be determined. A standard situation is that the protein, P, favours the bottom phase, whereas the ligand, L, favours the top phase. The bound ligand, PL, is then found in the bottom phase. So far, the situation is identical with the one in equilibrium dialysis. In partitioning, however, the amount of protein in the top phase must be considered. From these considerations a set of four equations can be used to describe the basic system.

The amount of free ligand in the bottom phase is obtained by rearrangement of eqn. 1 for the ligand:

$$C_{\text{L}}^b = C_{\text{L,tot}}^b / K_{\text{part,L}} \quad (4)$$

As an initial approximation, $C_{\text{L,tot}}^t$ is used instead of C_{L}^t in eqn. 4.

The amount of ligand bound to the protein in the bottom phase can then be expressed by

$$C_{\text{PL}}^b = C_{\text{L,tot}}^b - C_{\text{L}}^b \quad (5)$$

By making an analogous correction for the amount of ligand bound to protein in the top phase, eqns. 6 and 7 are obtained.

$$C_{\text{PL}}^t = C_{\text{PL}}^b \cdot K_{\text{part,PL}} \quad (6)$$

$$C_{\text{L}}^t = C_{\text{L,tot}}^t - C_{\text{PL}}^t \quad (7)$$

The new value for C_L^1 obtained in eqn. 7 can be used in eqn. 4, and the calculation should be repeated until all values are constant. Then we can use C_L^b and C_{PL}^b , *e.g.*, for the determination of the stoichiometry and the binding constant with a Scatchard plot¹⁰. In the Scatchard plots C_{PL}^b/C_P^b is plotted on the abscissa and $C_{PL}^b/(C_L^b \cdot C_P^b)$ on the ordinate.

If the partitioning of the reactants is studied for a system with a different partitioning behaviour of the reactants, *i.e.*, with the binding protein in the top phase and the ligand in the bottom phase, the superscripts *b* and *t* should be interchanged and the partitioning coefficients should be inverted in eqns. 4–7.

In a simple model system such as the one depicted above, eqns. 4–7 can be rearranged to give an equation system. Other systems may give more complicated equations that are more difficult or even impossible to rearrange in such a way. The method of making repeated calculations, starting from an approximation, is therefore a more general way of evaluating the experimental data.

The partition constant of the protein–ligand complex, $K_{part,PL}$, in eqn. 6 may not be known before the evaluation of an analysis. As an approximation, $K_{part,PL}$ can be replaced by $K_{part,P}$, which can be determined directly. A good estimation of $K_{part,PL}$ can be obtained indirectly in the following way. The apparent partitioning of the ligand is determined for different amounts of ligand or protein and a corresponding plot is made. For low ligand to protein ratios the curve will approach $K_{part,PL}$ asymptotically, as it can be assumed that most of the ligand is present as a protein–ligand complex under such conditions.

A listing of a computer program in Fortran IV or Basic, which performs the above calculations is available from the authors on request.

RESULTS AND DISCUSSION

The interaction between human serum albumin and Cibacron Blue was studied with equilibrium dialysis and partition affinity ligand assay (PALA). In both instances different amounts of the dye were added to a constant amount of albumin. The outcome of the equilibrium dialysis analysis is presented as a Scatchard plot in Fig. 1.

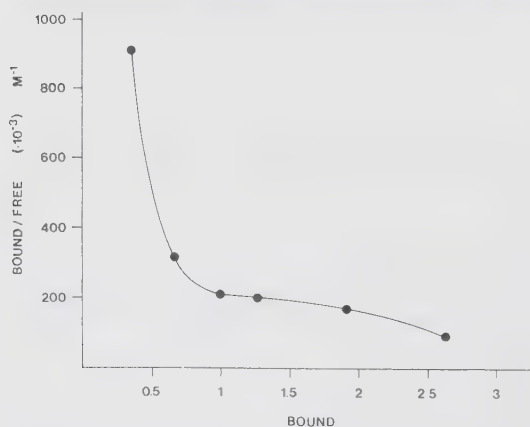


Fig. 1. Scatchard plot for the binding reaction between human serum albumin and Cibacron Blue using equilibrium dialysis to separate free and bound ligand.

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The result might be interpreted as a binding situation with several heterogeneous sites with different binding properties. The dye, however, adsorbed to the dialysis membrane, as do many other ligands of a hydrophobic nature. In this way a competitive binding situation arose where both the membrane and the protein competed for binding the ligand. The free ligand concentration used in the Scatchard equation should then be corrected for the amount of ligand adsorbed to the membrane. The association constant given by the Scatchard plot would, however, still be erroneous because the adsorption displaces the equilibrium of the protein-ligand interaction. When the result was recalculated with the free ligand concentration in the Scatchard equation replaced by the total amount of ligand added, the plot in Fig. 2 was obtained. As expected⁸, two molecules of Cibacron Blue could bind to one albumin molecule. The association constant was found to be $2.3 \cdot 10^4 \text{ l mol}^{-1}$ ($\pm 0.1 \cdot 10^4$, $2 \times \text{S.D.}$) which corresponds well with published values obtained by frontal analysis chromatography ($0.1 \cdot 10^4$ – $4.7 \cdot 10^4 \text{ l mol}^{-1}$)¹¹ and affinity electrophoresis ($0.56 \cdot 10^4 \text{ l mol}^{-1}$)¹². Fig. 2 thus gives a better description of the binding reaction than does Fig. 1, although a rather rough approximation is made. It is, however, difficult to make a strict comparison of association constants, as the dye is available only in a low grade of purity and the results may vary between different batches.

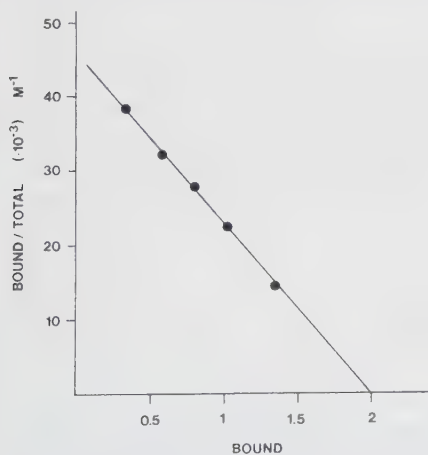
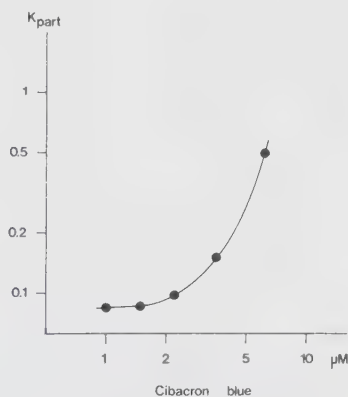


Fig. 2. Scatchard plot for the same system as in Fig. 1, but with the concentration of free ligand in the Scatchard equation replaced by the total concentration of ligand.

Fig. 3. The observed partitioning of Cibacron Blue in two-phase systems when different amounts of dye were mixed with a constant amount of human serum albumin. The concentration of albumin in the lower phase was 76 nM.



The same binding assay was studied in a two-phase system consisting of PEG and dextran. First the partitioning properties of the reactants were determined. Albumin had a partition coefficient of 0.137 and Cibacron Blue 7.79. The partitioning of the dye-albumin complex was determined by plotting the partitioning of the dye in the assay as a function of the dye concentration (Fig. 3). When the dye concentration is decreased the measured partitioning approaches that of the protein-ligand complex. In order to confirm the determination of the partition coefficient, the pro-

tein content in each phase was measured in a Kjeldahl analysis. Because of the comparatively low sensitivity, phase systems of 20 ml were used. Protein concentration determinations based on spectrophotometric measurements could not be used, owing to possible interference from the dye. The partitioning coefficients of the dye and the dye albumin complex were used in the evaluation of the binding analysis as described under Theoretical. An example of the calculations using eqns. 4-7 is given in Table I and the Scatchard plot obtained is shown in Fig. 4. It can clearly be seen that the plot is similar to that in Fig. 2; however, the association constant ($2.6 \cdot 10^4 \text{ l mol}^{-1}$; $\pm 0.1 \cdot 10^4$, $2 \times \text{S.D.}$) obtained is different. The discrepancies between the results obtained by the two methods may be explained by the fact, that the values for equilibrium dialysis (Fig. 2) are calculated using an approximation that does not account for the fact that the adsorption phenomenon may affect the association constant.

TABLE I

CALCULATION OF THE CONCENTRATION OF FREE AND BOUND LIGAND FOR ONE OF THE POINTS IN FIG. 3, USING EQNS. 4-7

Iteration number*	Free ligand (M)	Bound ligand (M)	Scatchard	
			"Y"	"Y"/free (l mol ⁻¹)
1	$5.76 \cdot 10^{-6}$	$1.30 \cdot 10^{-4}$	1.715	$2.98 \cdot 10^5$
2	$3.87 \cdot 10^{-6}$	$1.32 \cdot 10^{-4}$	1.740	$4.49 \cdot 10^5$
3	$3.84 \cdot 10^{-6}$	$1.32 \cdot 10^{-4}$	1.740	$4.52 \cdot 10^5$
4	$3.84 \cdot 10^{-6}$	$1.32 \cdot 10^{-4}$	1.740	$4.52 \cdot 10^5$

* The iterations were initiated with the following values:

Partition coefficient of Cibacron Blue: 7.79

Partition coefficient of complex: 0.085

Concentration of albumin: $7.60 \cdot 10^{-5} \text{ M}$

Concentration of ligand in bottom phase: $1.36 \cdot 10^{-4} \text{ M}$

Concentration of ligand in top phase: $4.49 \cdot 10^{-5} \text{ M}$

Another phase system, consisting of PEG and magnesium sulphate, was also used for the separation of bound and free ligand. This phase system gave a more efficient separation; K_{part} for albumin was 0.025 and for Cibacron Blue 11.4, and consequently the system caused some dissociation of the protein-dye complex. A binding analysis was made and a Scatchard plot similar to that obtained with the PEG dextran system was found. However, measurements with low concentrations of the dye were difficult to perform and subject to significantly increased experimental errors.

The binding analysis described above could be utilized as a basic set-up in different assays. In a direct binding assay, the partitioning of a constant amount of Cibacron Blue was measured and plotted as a function of the amount of serum albumin present (Fig. 5). The binding of various ligands could be measured in competitive binding assays, where the ligand and Cibacron Blue competed for the binding to albumin as exemplified in Fig. 6.

From the practical point of view, the difference between equilibrium dialysis

COMPARISON BETWEEN BINDING ANALYSES

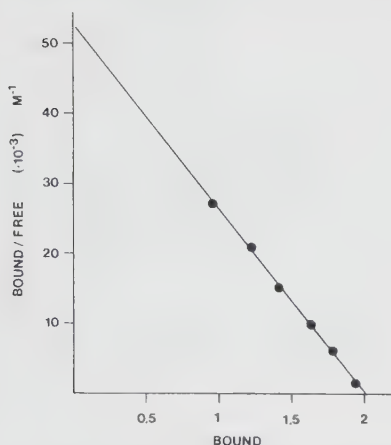


Fig. 4. Scatchard plot for the binding reaction between human serum albumin and Cibacron Blue. Free and bound ligand were separated by partitioning in two-phase systems consisting of PEG and dextran. The concentration of albumin in the lower phase was 76 nM.

and PALA is obvious. The former procedure required 24 h to reach equilibrium and one dialysis cell is required for each determination. The PALA procedure required 1 h, including the time for evaluation, and the binding and separation take place in standard test-tubes. Further, PALA has potential for the analysis of interactions between macromolecules and cells and other reactant pairs that are difficult to handle by conventional methods.

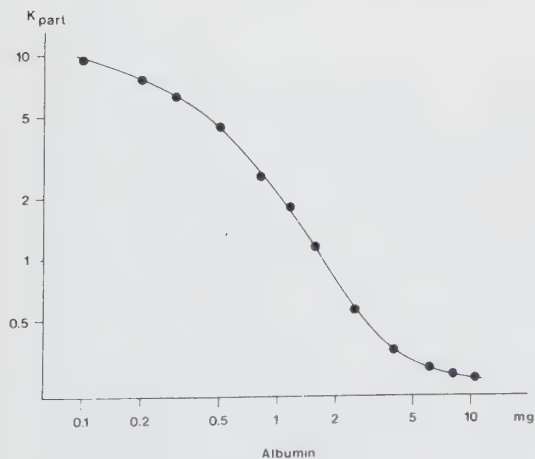
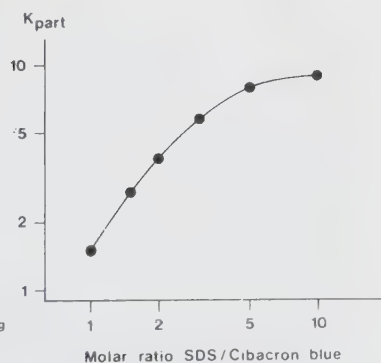


Fig. 5. Direct binding assay of human serum albumin. The partitioning of a constant amount of Cibacron Blue was measured as a function of the amount of albumin present in the system.

Fig. 6. Competitive binding assay of sodium dodecyl sulphate (SDS). SDS and Cibacron Blue competed for the binding to human serum albumin. The partitioning of a constant amount of Cibacron Blue was measured as a function of the amount of SDS present in the system.



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A General Study of the Binding and Separation in Partition Affinity Ligand Assay. Immunoassay of β_2 -Microglobulin

T.G.I. Ling and B. Mattiasson

Department of Pure and Applied Biochemistry, Chemical Center, P.O. Box 740, S-220 07 Lund, Sweden

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Partitioning in aqueous 2-phase systems was used to separate free and bound ligand in an immunoassay for β_2 -microglobulin. In order to get efficient separation in the phase system, the antibodies were modified to favour their partition in a different phase from that of antigen. However such modification of antibodies significantly decreased their binding capacity. This was overcome by using antibodies bound to previously modified staphylococci, which had proper partitioning behaviour. Alternatively, antibodies conjugated with biotin could be used in combination with modified avidin.

This paper presents a method for the evaluation of data from immunoassays whereby 2-phase systems have been used to separate free and bound antigen.

Key words:

Introduction

Separation of free and bound ligand in immunoassays by means of aqueous 2-phase systems has been found to be a promising alternative to other separation techniques (Mattiasson and Ling, 1980). The principle of separation in 2-phase systems is the partition of antigen to one of the phases and antibodies and the antigen-antibody complex to the other phase. In this way binding is determined as translocation of labelled antigen from one phase to the other, since it changes partitioning behaviour when bound to antibody. In practice it is sufficient that only one of the two reactants undergoes partition asymmetrically. Partitioning in aqueous 2-phase systems is rapid and reproducible technique for separating various compounds and has been used in several binding assays (Shanbhag et al., 1973; Mattiasson, 1980; Mattiasson and Ling, 1980; Mattiasson et al., 1981; Ling et al., 1982; Mattiasson and Eriksson, 1982; Urios et al., 1982).

A crucial problem that occurs with the use of this method is insufficient asymmetry in the partition pattern. In the present paper we present 2 different methods employed to overcome this. Assay of β_2 -microglobulin was chosen as a

model since it could be assumed that antigen and antibodies would partition in a similar way owing to the homology in their structures. A general approach is presented here that might be followed in devising a partition affinity ligand assay (PALA).

Materials and Methods

QAE-Sephadex® A-50 and Sepharose® CL-4B were purchased from Pharmacia Fine Chemicals (Uppsala). A radioimmunoassay kit directed against β_2 -microglobulin (Phadebas®, β_2 -micro test) was obtained from Pharmacia Diagnostics AB (Uppsala). Native and ^{125}I -labelled β_2 -microglobulin were used as supplied in the kit. Antiserum against β_2 -microglobulin and heat-treated and formaldehyde-stabilized *Staphylococcus aureus* Cowan I were a kind gift from Pharmacia Diagnostics AB. Polyethylene glycol 4000, PEG-4000, and triazine came from BDH (Poole). Monomethoxy-polyethylene glycol, M-PEG, with molecular weights of 2000 and 5000 respectively were generous gifts of Union Carbide (New York). 1,6-Diaminohexane was obtained from Fluka AG (Buchs). Horseradish peroxidase type VI, biotin and 2(4'-hydroazobenzene) benzoic acid (HABA) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade.

Purification of avidin

Purification of avidin from egg white was by affinity chromatography with HABA-Sepharose (Bayer and Wilchek, 1974). Sepharose CL-4B was activated with 0.1 g CNBr per ml gel (Axén et al., 1967) and 1,6-diaminohexane was added in large excess. After washing with 1 litre of 1 M NaCl, 0.1 M NaHCO_3 , pH 8.3, 8.0 μmol HABA was added per ml gel. Egg white (5 ml) was mixed gently with 45 ml 0.5 M NaCl, 0.10 M sodium phosphate, pH 7.00, and passed through a column containing 25 ml HABA-Sepharose. Avidin bound to the gel and was eluted with 0.2 M acetic acid, pH 2.8.

Biotinyl-peroxidase

This was prepared by coupling biotinyl-1,6-diaminohexane to horseradish peroxidase according to Wilson and Nakane (1978).

Purification of antibodies

Antibodies were purified from antiserum by ion exchange chromatography on QAE-Sephadex with sodium phosphate buffer, pH 7.00, ionic strength 0.1 M. The antibodies passed through the resin unretarded while most other serum proteins were adsorbed.

Modification procedure

Monomethoxy-polyethylene glycol, M-PEG-5000, was activated with triazine (Abuchowski et al., 1976). Antibodies were modified by adding 50 mg activated M-PEG to 1 ml of antibodies (1 mg/ml) in 0.1 M triethanolamine-HCl, pH 9.4,

followed by incubation for 2 h at room temperature on a rocking table. *Staphylococcus aureus* cells were modified according to the same procedure, with 10 mg activated M-PEG to 1 ml of a suspension of bacteria containing 3×10^9 cells/ml. Avidin was modified in the same way, with 50 mg M-PEG-2000 to 5 mg avidin in 1 ml.

Assay procedure

The reactants were mixed in a test tube and buffer was added to a final volume of 100 μ l. The buffer consisted of 90 mM KCl and 10 mM Tris-HCl, pH 7.50 and was used for all dilutions. After an incubation time of 20 min, 900 μ l of a well-mixed phase system containing 0.15 g/g PEG-4000 and 0.15 g/g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ was added. After separation had occurred (15 min), an aliquot of 200 μ l was removed from each phase for determination of radioactivity or enzyme activity. The amount of material adsorbed to the interface was calculated by subtracting the activity found in each phase from the total activity added. The partition coefficient is defined as the ratio between the concentration in the top and bottom phase, respectively:

$$K_{\text{part}} = C_{\text{top}}/C_{\text{bottom}}$$

When modified *S. aureus* were used, the antiserum was preincubated with the cells (0.25 ml antiserum to 4×10^8 cells) for 1 h at room temperature on a rocking table and unbound material was washed off. The cells with adsorbed antibodies were used immediately or stored at 4°C.

Results and Discussion

In an immunoassay by the PALA technique, one of the first steps is to establish proper separation conditions. In order to find a suitable phase system, the partitioning behaviour of the reactants must be studied, since there are many factors that can influence the partition coefficient (Albertsson, 1971). Not only the type of polymers or salts that make up the phase system govern the partitioning of a molecule, but also other important factors including the length of the polymers used in the phase system, the pH and the presence of different ions.

The partitioning coefficients of β_2 -microglobulin and rabbit antibodies, respectively, were determined for 2 phase systems with quite different properties and with different ions that gave interfacial potentials (Eriksson and Johansson, 1979) of opposite signs (Table I). The 2 proteins were found to have very similar partition behaviour and no conditions for their effective separation were found. The partition coefficient of β_2 -microglobulin was consistently found to be about 1.7 times that of the antibodies for the 6 phase systems listed in Table I. The antibodies were slightly more sensitive to variations of the interfacial potential as can be seen from the difference in K_{part} for different buffers. This similarity in partitioning was not unexpected, since the structure of β_2 -microglobulin is very similar to the constant regions of the γ -chain in human immunoglobulin G (Peterson et al., 1972).

TABLE I

PARTITION COEFFICIENTS OF β_2 -MICROGLOBULIN AND ANTI- β_2 -MICROGLOBULIN IN DIFFERENT PHASE SYSTEMS

The PEG/dextran system consisted of 0.060 g PEG-6000 per g phase system and 0.10 g/g Dextran T-70. The PEG/salt system consisted of 0.15 g/g PEG-4000 and 0.15 g/g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$. The buffers were: phosphate, 0.10 M sodium phosphate pH 7.00; Tris-HCl, 0.10 M Tris-HCl, pH 7.50; Tris-KCl, 0.010 M Tris-HCl, pH 7.50, 0.090 M KCl. For the determinations, 100 μl of protein in the indicated buffer were mixed with 900 μl of the phase system and the mixture allowed to separate for 30 min before determination of protein concentration in each phase.

Phase system	Buffer	Partition ($\beta_2\text{m}$)	Partition (anti- $\beta_2\text{m}$)
PEG/dextran	Phosphate	1.71	1.05
	Tris-HCl	1.24	0.74
	Tris-KCl	1.13	0.60
PEG/salt	Phosphate	0.53	0.33
	Tris-HCl	0.51	0.32
	Tris-KCl	0.50	0.30

One way to change the partitioning of a molecule is to modify its surface properties. This can be achieved by covalent attachment of a polymer with hydrophobic/hydrophile properties so that it partitions predominantly to one of the phases in the phase system. The modification has previously been effected by using monomethoxy-polyethylene glycol, M-PEG (Mattiasson and Ling, 1980; Mattiasson et al., 1981), to make the molecule partition to the PEG-rich top phase. Alternatively, antibodies have been immobilized on Sephadex particles that partition to the bottom phase (Mattiasson, 1980).

A phase system consisting of PEG-4000 and MgSO_4 was chosen for this study. β_2 -Microglobulin partitioned slightly to the bottom phase (Table I), so the antibodies were modified with M-PEG to make them partition to the top phase.

When antibody is heavily modified its binding capacity may decrease, probably due to steric hindrance of the binding site. It is therefore insufficient to determine the partition coefficient for the antibody before and after modification in order to measure the separation effect; the ability of the binding protein to transport bound labelled antigen across the phase boundary must also be measured. This transport capacity is maximal when the proper degree of modification is achieved (Mattiasson and Ling, 1980; Mattiasson et al., 1981).

M-PEG modified antibody lost a significant degree of binding capacity even with a rather mild degree of modification that did not alter its partitioning properties enough to enable it to transport bound antigen to the top phase and thereby separate bound and free antigen into different phases. Fig. 1 shows the titration of transport capacity for ^{125}I -labelled β_2 -microglobulin of the batch of M-PEG antibodies used in subsequent experiments. The amount of antibody used in the assay

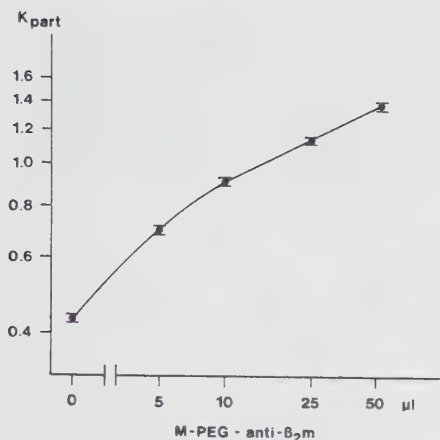


Fig. 1. Transport capacity of M-PEG anti- β_2 -microglobulin in moving iodine-labelled β_2 -microglobulin from the bottom phase to the top phase. The partition coefficient of the labelled antigen is plotted as a function of the amount of M-PEG antibody added.

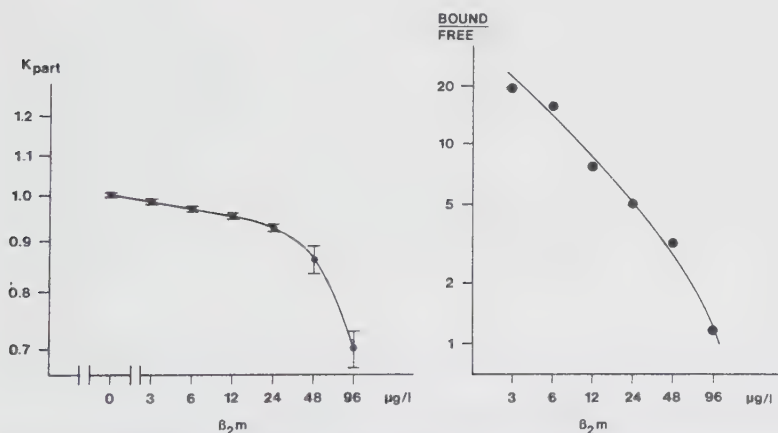


Fig. 2. Standard curve for a competitive immunoassay of β_2 -microglobulin. Bound and free antigen were separated by partitioning in 2-phase systems. A 25 μ l 125 I-labelled β_2 -microglobulin (6.0 μ l/l) and 150 μ l β_2 -microglobulin in varying concentrations were mixed with M-PEG antibody (0.92 mg/ml) and the mixture incubated for 15 min. Then 1.8 ml of the PEG/MgSO₄ phase components was added and the samples were thoroughly mixed. After 10 min, an aliquot of 400 μ l was taken from each phase for determination of radioactivity. a: The partition coefficient of the labelled antigen is plotted as a function of the concentration of added antigen. b: the same data as in a recalculated to give bound/free ratios by using the equations given in the Appendix. The bound/free ratio without added β_2 -microglobulin added was 52.0.

was a compromise between the amount required for good transport capacity and the amount which would lead to a decrease in sensitivity of the method.

When modified antibody was used in a binding assay, a standard curve was obtained as shown in Fig. 2a. The range of the partition coefficients was small in comparison to that obtained in previous assays, but the reproducibility was good (relative standard deviation = 0.05). Since both free and bound labelled β_2 -microglobulin was found in both phases, the partitioning coefficient does not indicate the bound/free ratio directly. When the values in Fig. 2a were recalculated to get the bound/free ratio (Fig. 2b) it was clear that the assay performed well despite the poor separation. The calculations used are given in the Appendix.

The results also show that a moderate difference in partition coefficient between the reactants may be sufficient for their use in a binding assay. A marked difference should lead to an effective separation process and, in theory, might give rise to a situation where all free antigen would appear in one phase and all the complex-bound antigen in the other. Since binding is a reversible reaction such elimination of free reactant might lead to dissociation of the antigen-antibody complex. This possibility is especially relevant when reactant pairs with low binding constants are used. This risk, however, has proved to be negligible under the conditions normally used in the PALA procedure. This risk is present in all binding assays and when weak interactions are under study it is important that separation is fast and reproducible, so that no unwanted release of bound antigen takes place during the separation process.

In attempt to obtain a more general and gentle solution to the separation problem in immunoassay with the PALA technique, antibody was first attached to a separator, that is a molecule or particle with appropriate partitioning behaviour. Avidin and *Staphylococcus aureus* cells, respectively, were used as separators. Both separators may be modified to enable them to partition to either the top or the bottom phase (Table II).

S. aureus cells have protein A that binds to the Fc part of immunoglobulin G (Goding, 1978) leaving the antigen-binding Fab part free and oriented outwards from the cell surface. *S. aureus* has previously been used in place of a second antibody in immunoassays (Natali et al., 1979). *S. aureus* were first modified with

TABLE II

DISTRIBUTION IN TWO-PHASE SYSTEMS FOR THE SEPARATORS AVIDIN AND *Staphylococcus aureus*

A phase system consisting of 0.15 g/g PEG-4000 and 0.15 g/g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ was used.

	<i>Staphylococcus aureus</i>		Avidin M-PEG- modified
	Native	M-PEG- modified	
Top phase	0%	80%	90%
Interface	10%	20%	0%
Bottom phase	90%	0%	10%

M-PEG to make them partition to the top phase and then the antibody was added. In this way the antibody itself did not suffer chemical treatment. Furthermore, one *S. aureus* cell has around 80,000 protein A molecules on to surface (Kronvall and Frommel, 1970), so the risk of a deleterious loss of binding capacity during the modification step may be discounted. A check was, however, made that the *S. aureus* cells were not loaded with antibody, which might have altered their preferential

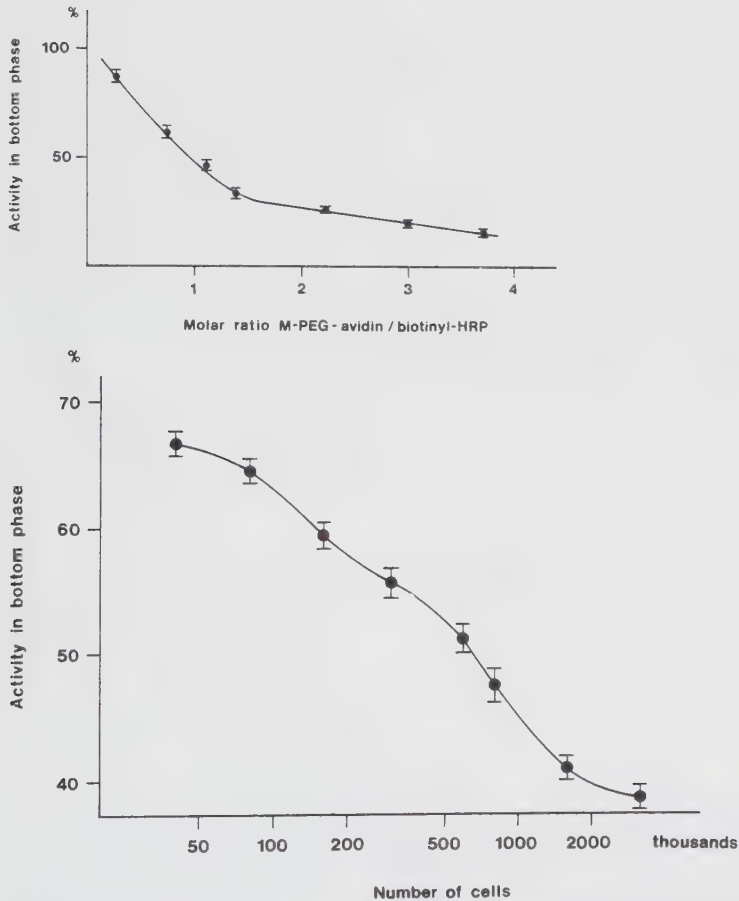


Fig. 3. Transport capacity of 2 separators in moving bound material from the bottom phase to the top phase. a: M-PEG-avidin used as a separator and binding to biotinylated peroxidase (80 μ g). b: M-PEG-modified *S. aureus* used as a separator and binding iodine-labelled antibody (50 μ g). The activity in the bottom phase is plotted as a function of the amount of separator added. 100% corresponds to the activity without separator present.

partitioning to the top phase. In the present study conditions were chosen so that no such effect was observed.

An alternative to *S. aureus* as a separator is the avidin/biotin system. In this case, the antibody is conjugated with biotin and the avidin is M-PEG modified to enable it to partition to the top phase (Table II). The conjugation of antibodies with biotin does not decrease their binding capacity, since biotin is a very small molecule and does not impose steric hindrance on the binding site. Biotinylated antibodies and lectins are commercially available.

the partitioning coefficients of *S. aureus* and avidin as separators are given in Table 2, and show the applicability of the system. More important than the partitioning, however, is the transport capacity of the separator. Fig. 3. shows the transport capacities of M-PEG-modified *S. aureus* and M-PEG-avidin. The association constant for the interaction between biotin and avidin is about 10^{15} M^{-1} , which makes the binding virtually irreversible. The complex M-PEG-avidin and biotin is thus very stable and M-PEG-avidin need not to be added in large excess. The first linear part of the curve in Fig. 3a shows that the transport capacity is directly proportional to the amount of M-PEG-avidin added. However, above a certain concentration of M-PEG-avidin the efficiency decreases, probably due to the presence of enzyme molecules lacking clearly exposed biotin. The biotinylated peroxidase was not purified by affinity chromatography, since 6 M guanidine-HCl, pH 1.0, is required for dissociation of the biotin-avidin complex and this condition clearly causes loss of enzyme activity.

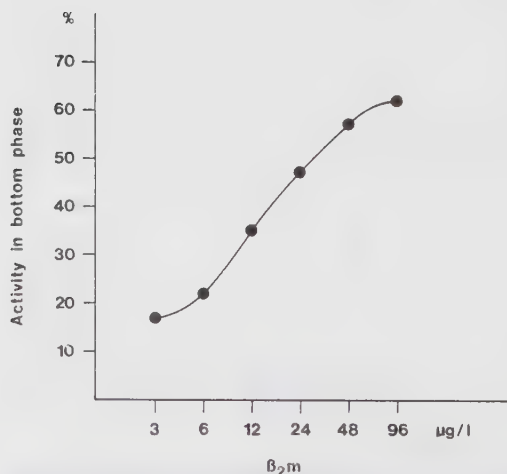


Fig. 4. Standard curve for a competitive assay of β_2 -microglobulin. M-PEG-modified *S. aureus* were used as separators and moved the antibodies to the top phase and the interface. Otherwise, the same conditions were used as in Fig. 2. In the bottom phase only free antigen was found. The amount of labelled antigen in the bottom phase is plotted as a function of the amount of antigen added.

In the PEG/MgSO₄ phase system a significant proportion of the modified cells were adsorbed at the interface, and thus the antibodies were found in the top phase and at the interface. Fig. 3b shows the capacity of M-PEG-modified *S. aureus* to move antibodies from the bottom phase. In the assay the cells were added in large excess, so the bottom phase contained free antigen only. A standard curve was obtained for this (Fig. 4). Since the partitioning coefficient was not applicable in this case, the activity in the bottom phase was used for determining the degree of binding. The concentration range obtained was the same as for the Pharmacia kit.

The use of avidin or *S. aureus* cells as separators provides a general and practical solution to the separation problem in assays where both antigen and antibody partition to the same phase and where modification of either of the reactants is undesirable. It also simplifies preparations for assay, since the separator can be stored and no further chemical treatment is required. Where it is desired to have the antibody in the bottom phase, it can be bound to native *S. aureus* which go to the bottom phase in most 2-phase systems.

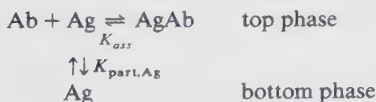
It is also possible to evaluate binding data when no definite separation occurs by theoretical treatment that allows use of PALA to study weak interactions between reactants or cases where the reactants have similar partitioning behaviour. PALA can then be used as an easy and rapid alternative to equilibrium dialysis.

Appendix

The partitioning of a molecule in a phase system is characterized by the partitioning coefficient, K_{part} , defined as:

$$K_{\text{part}} = C_{\text{top}}/C_{\text{bottom}} \quad (1)$$

In partition affinity ligand assay, an antigen, Ag, is present in a 2-phase system together with an antibody, Ab. In the following the superscript, t, denotes the top phase and b the bottom phase.



if the partitioning of labelled antigen is measured in a competitive immunoassay, the partition coefficient will be approximately equal to the bound/free ratio. In some cases, however, the amount of antibody and antigen-antibody complex in the bottom phase must be taken into account. A set of 4 equations may be used to compensate for insufficient separation in the phase system.

The amount of free antigen in the bottom phase is obtained by rearrangement of equation (1) for the antigen:

$$C_{\text{Ag}}^{\text{b}} = C_{\text{Ag}}^{\text{t}}/K_{\text{part, Ag}} \quad (2)$$

As an initial approximation $C_{Ag, tot}^t$ is used instead of C_{Ag}^t in eq. (2).

The amount of antigen-antibody complex in the bottom phase can then be expressed:

$$C_{AgAb}^b = C_{Ag, tot}^b - C_{Ag}^b \quad (3)$$

By making an analogous correction for the amount of antigen-antibody complex in the top phase, eq. (4) and (5) are obtained.

$$C_{AgAb}^t = C_{AgAb}^b * K_{part, AgAb} \quad (4)$$

$$C_{Ag}^t = C_{Ag, tot}^t - C_{AgAb}^t \quad (5)$$

The new value for C_L^t obtained in eq. (5) can be used in eq. (2) and the calculation should proceed until all values are constant.

We now find the total amount of free and bound antigen:

$$Free = C_{Ag}^t + C_{Ag}^b \quad (6)$$

$$Bound = C_{AgAb}^t + C_{AgAb}^b \quad (7)$$

If the partitioning of the reactants is studied for a system with different partitioning behaviour of the reactants, i.e., with antibody in the top phase and antigen in the bottom phase, the superscripts b and t should be interchanged and the partitioning coefficients should be inverted in eq. (2)-(5).

A Listing of a computer program which performs the above calculations is available on request in FORTRAN IV or BASIC.

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POLY(ETHYLENE GLYCOL)- AND POLY(VINYL ALCOHOL)-SUBSTITUTED CARBOHYDRATE GELS FOR "MILD" HYDROPHOBIC CHROMATOGRAPHY*

T. G. I. LING* and B. MATTIASSON

Pure and Applied Biochemistry, Chemical Centre, University of Lund, P.O. Box 740, S-220 07 Lund (Sweden)

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SUMMARY

The possibility of using polymers with intermediate hydrophobicity as ligands in hydrophobic interaction chromatography was studied. Serum albumin and haemoglobin were separated on a column using poly(vinylalcohol)-Sephacrose. The retention of haemoglobin increased with increasing temperature or increasing ionic strength, indicating that the interaction with the stationary phase was of a hydrophobic nature. The retention of albumin was, however, relatively unaltered.

Glucose oxidase was modified by covalent attachment of monomethoxypoly(ethylene glycol) and conjugation with gentamicin. Two fractions were obtained when the product was run on monomethoxypoly(ethylene glycol)-Sephadex at high ionic strength. The hydrophobicities of the two fractions were measured by partitioning in an aqueous two-phase system.

INTRODUCTION

It is possible to separate successfully biomolecules chromatographically by exploiting their hydrophobic properties. Hydrophobic moieties such as alkyl and phenyl groups have been extensively examined as ligands in chromatography. Many proteins have been found to bind to these ligands, thus permitting chromatographic separation. However, negative side effects have also been noted. The binding is, in many cases, too strong to be useful in a chromatographic process and may even be practically irreversible¹⁻³. Furthermore, in order to elute a strongly bound protein, chaotropic agents or organic solvents may be required, which can lead to denaturation. Ligands having an intermediate hydrophobicity would thus be of interest, since they would permit adequate binding strength without the above-mentioned disadvantages.

Weak hydrophobic interactions are, however, used in another separation

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method, *viz.* partitioning in an aqueous two-phase system. These systems are composed of two aqueous solutions, either two polymers or one polymer and a salt. Examples of such systems are poly(vinyl alcohol)-dextran, poly(ethylene glycol)-dextran, poly(ethylene glycol)-magnesium sulphate and methylcellulose dextran. Aqueous two-phase systems have been utilized to separate many kinds of biomolecules⁴. As it is possible to get separation effects when there are comparatively small differences in hydrophobicity between the two aqueous phases, it seemed probable that separation could also be obtained if the same polymers were used as chromatographic materials.

The analogy between partitioning in aqueous two-phase systems and chromatographic procedures has previously been utilized in partition chromatography⁵. In this case, one of the two phases was merely adsorbed to the stationary phase and the experimental conditions were adjusted so that the adsorbed phase constituent was not dissolved.

We have used hydrocarbons substituted with hydroxy or ethergroups, such as poly(ethylene glycol) and poly(vinyl alcohol) covalently bound as ligands, in order to obtain "mild" hydrophobic chromatography. The material to be separated consisted of mixtures of pure proteins as well as derivatized proteins with slightly modified hydrophobicities.

MATERIALS AND METHODS

Bio-Gel P-4 was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Sephadex G-25, Sepharose 6B and epoxy-activated Sepharose 6B were obtained from Pharmacia (Uppsala, Sweden). Poly(ethylene glycol) (PEG-4000, new designation PEG-3350) came from Union Carbide (New York, NY, U.S.A.). Monomethoxypoly(ethylene glycol) (M-PEG) with a molecular weight of 5000 was a generous gift from Union Carbide. Poly(vinyl alcohol) (PVA), molecular weight 13000, was purchased from Serva (Heidelberg, G.F.R.). Bovine serum albumin, bovine haemoglobin, whale myoglobin, horseradish peroxidase (type VI) (E.C. 1.11.1.7), glucose oxidase (type V) (E.C. 1.1.3.4), N-hydroxysuccinimide, 4-aminoantipyrine and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were obtained from Sigma (St. Louis, MO, U.S.A.). Gentamicin was a generous gift from Schering (Kenilworth, NJ, U.S.A.). All other chemicals used were of analytical-reagent grade.

Preparation of "mild" hydrophobic resins

M-PEG-Sephadex G-25 Medium. M-PEG was activated with epichlorohydrin to make a glycidyl ether which was then coupled to Sephadex^{6,7}.

PVA-Sepharose. PVA (4 g) in 0.1 *M* sodium carbonate (pH 11.0) (1 l) was coupled to epoxy-activated Sepharose 6B (9 ml) according to a procedure described by Pharmacia⁸.

Modification of proteins with monomethoxy-PEG (M-PEG)

Activation of M-PEG with triazine was performed according to the method of Abuchowski *et al.*⁹.

Glucose oxidase (1.5 mg) was dissolved in a solution of 0.1 *M* triethanolamine hydrochloride and 0.1 *M* sodium chloride (pH 9.4) and dialysed against the same

buffer. The enzyme was modified by the addition of 40 mg of activated M-PEG (in a total volume of 5 ml) and incubated for 1 h on a rocking table at room temperature. Then a conjugate between M-PEG–glucose oxidase and gentamicin was formed using a carbodiimide coupling procedure. N-Hydroxysuccinimide (1.15 mg) and EDC (1.9 mg) were added to the modified protein and after 15 min gentamicin (0.22 mg) was also added. Finally, glycine was added to give a concentration of 1.0 M.

Glucose oxidase activity was measured by a two-step enzymatic reaction where glucose oxidase oxidized glucose giving gluconic acid and hydrogen peroxide, which then acted as a substrate for peroxidase. The final concentrations were: 1.25 $\mu\text{g/ml}$ horseradish peroxidase, 45.4 mM glucose, 1 mM 4-amino-antipyrine and 17.5 mM phenol. The enzyme activity was determined spectrophotometrically at 510 nm.

Two-phase systems. The protein solution (100 μl) was mixed with a well-mixed phase system (900 μl) to give a final concentration of 0.135 g/g PEG-4000, 0.135 g/g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 10 mM Tris–HCl (pH 7.50). The partitioning coefficient is defined as the ratio of the concentration in the top and bottom phase, respectively:

$$K_{\text{part}} = C_{\text{top}}/C_{\text{bottom}}$$

RESULTS AND DISCUSSION

The following characteristics have been proposed as criteria for hydrophobic interaction chromatography: (1) hydrophobic sites can be identified on the stationary phase; binding is promoted by (2) increased temperature and (3) increased ionic

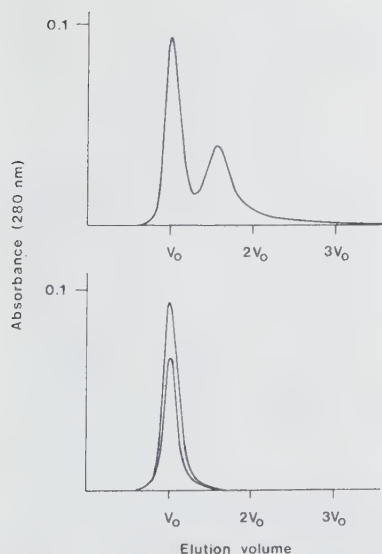


Fig. 1. (a) Separation of bovine serum albumin and bovine haemoglobin on a column (25 $\times$ 0.7 cm) of PVA–Sephrose at 26 $^{\circ}\text{C}$ and 4.8 ml/h. The mobile phase consisted of 3 M sodium chloride and 10 mM Tris–HCl (pH 7.50). A 30- μl sample was applied, containing 17 mg/ml of albumin and 6 mg/ml of haemoglobin. (b) Same conditions as in (a) but using unsubstituted Sepharose.

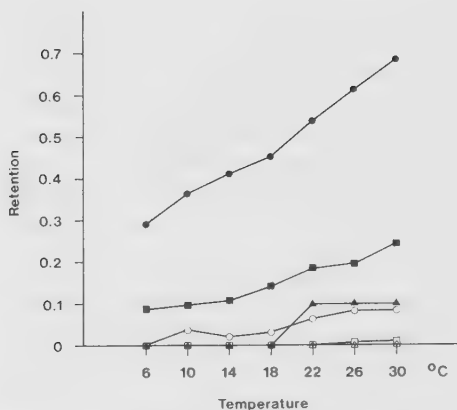


Fig. 2. Retention of serum albumin and haemoglobin as a function of temperature and ionic strength. Experimental conditions are given in Fig. 1a. For 0.15 *M* sodium chloride, the retention of both proteins is zero over the whole temperature range. Retention is defined as $(V_e - V_0)/V_0$, where V_e is the elution volume and V_0 is the void volume for the proteins. Symbols used: albumin at (△) 1 *M*, (□) 2 *M* and (○) 3 *M* sodium chloride; haemoglobin at (▲) 1 *M*, (■) 2 *M* and (●) 3 *M* sodium chloride.

strength¹⁰. All three points have to be met in order to establish the hydrophobic nature of the interactions.

The above criteria were used to evaluate the chromatographic separation of bovine serum albumin and bovine haemoglobin on PVA Sepharose. It was found that the retention time for albumin did not depend very much on the ionic strength or the temperature, whereas haemoglobin was retarded when the ionic strength or temperature was increased implying that its interaction was of a hydrophobic nature. Fig. 1a shows an example of a chromatogram. The peaks were identified by making runs for each protein separately. The retention values of the two proteins are shown in Fig. 2 as a function of temperature and ionic strength. As a control, the two proteins were run on PVA-Sepharose at physiological ionic strength (0.15 *M*) and on unsubstituted Sepharose using 3 *M* sodium chloride at 26°C. In both cases a single symmetrical peak was obtained (Fig. 1b). In no case was material eluted when the ionic strength was decreased to 0.15 *M*, after a run with an ionic strength in the range 1–3 *M*.

The results differ markedly from those obtained on conventional hydrophobic interaction chromatography with, *e.g.*, octyl-Sepharose, where albumin is strongly retarded³. Serum albumin has binding sites for fatty acids and other hydrophobic ligands¹¹ which may be responsible for its binding to alkyl-gels. Such specific interactions are not expected when albumin is run on gels substituted with PVA or PEG. The results also demonstrate that aside from those specific hydrophobic regions, the molecules are hydrophilic in nature.

An important factor in hydrophobic interaction chromatography is the solubility of the proteins to be separated. It is generally known that adsorption on to a hydrophobic gel is expected to occur when the ionic strength is increased to a point rather close to that for salting-out precipitation of the protein. Since albumin has a good solubility even at 3 *M* sodium chloride, it may be expected that albumin would not be retarded on the PVA-Sepharose gel, a prediction that holds (Fig. 2).

GELS FOR HYDROPHOBIC CHROMATOGRAPHY

TABLE I

RETENTION TIMES FOR THREE PROTEINS ON PVA-SEPHAROSE RUN AT 26°C AND 3 M SODIUM CHLORIDE

Myoglobin was eluted later than albumin and haemoglobin at low ionic strength, probably because of a gel filtration effect. In the corrected retention, the elution volume of myoglobin at 0.15 M sodium chloride was defined as the void volume.

Protein	Relative retention	Corrected retention
Bovine serum albumin	0.07	0.07
Bovine haemoglobin	0.62	0.62
Whale myoglobin	0.35	0.20

The other component in the mixture is a tetrameric protein, haemoglobin. This protein is known to dissociate into dimers at high ionic strengths¹². The dimeric and tetrameric forms are in a rapid equilibrium, which can be followed by measuring the apparent molecular weight. When the ionic strength is increased from 0.15 to 3 M by addition of sodium chloride, the apparent molecular weight is slightly decreased. This effect is, however, too small to cause a separation based on gel filtration¹³. The fact that no separation occurs when the two proteins are run on unsubstituted Sepharose with 3 M sodium chloride also indicates that the separation is not dependent on the dissociation *per se*.

The solubility of haemoglobin is significantly lower than for serum albumin but high enough to show that the retention cannot be explained merely as an effect of limiting solubility. This is also shown by the fact that the same chromatographic behaviour was obtained when the protein solution was diluted ten times before application on to the column. Furthermore, myoglobin was run as indicated in Table I. Myoglobin is more soluble than both haemoglobin and albumin, but had an intermediate retention.

PEG and PVA are generally regarded as hydrophilic polymers, as they are soluble in water, in contrast to many other polymers. However, hydrophilicity and hydrophobicity are, in this context, no strict terms but merely relative notations. The

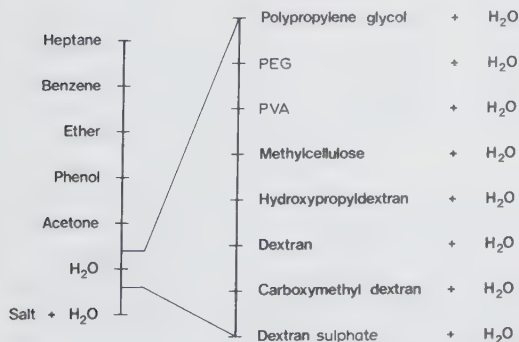


Fig. 3. Hydrophobic scale, where the different hydrophobicities of aqueous solutions are indicated for different polymers. (Reproduced from Albertsson⁴ by kind permission.)

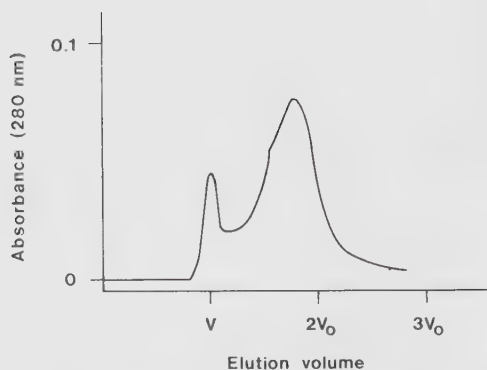


Fig. 4. Chromatogram showing the separation of M-PEG-gentamicin-glucose oxidase into two fractions on a M-PEG-Sephadex G-25 column (32×1.0 cm). The mobile phase consisted of 1.41 *M* magnesium sulphate and 10 *mM* Tris-HCl (pH 7.50).

fact that some polymers are water soluble does not prove that they cannot have hydrophobic properties. Aqueous solutions of different polymers can be ordered in a hydrophobic scale in the same way as organic solvents⁴, as shown in Fig. 3.

The project for using these mild chromatographic systems was initiated from a need to separate native from modified proteins, which were prepared for use in partition affinity ligand assay¹⁴. At that time no useful chromatographic technique was available for separating proteins which differed only by the presence or absence of a few chains of covalently attached poly(ethylene glycol). For the above-mentioned approach to be useful, there must be a correlation between partition behaviour in the phase system and the corresponding chromatographic behaviour on the column. For this reason the hydrophobicity of poly(ethylene glycol) modified glucose oxidase was measured using these two methods and the two sets of results were compared subsequently.

We have previously attached PEG to proteins in order to govern their partitioning in aqueous two-phase systems. For example, native concanavalin A (Con A) partitions to the salt-rich bottom phase in the PEG-magnesium sulphate phase system ($K_{\text{part}} = 0.031$) whereas PEG-Con A partitions to the PEG-rich phase ($K_{\text{part}} = 80$)¹⁴. In this study, however, glucose oxidase was used. Native glucose oxidase had a K_{part} of 0.077 in the PEG-magnesium sulphate system as determined from the enzyme activity. The M-PEG-modified and gentamicin-conjugated glucose oxidase had a K_{part} of 0.064. This is lower than for the native enzyme, probably because of the hydrophilic nature of gentamicin.

The M-PEG-modified and gentamicin-conjugated glucose oxidase was run on M-PEG Sephadex (Fig. 4) with 1.41 *M* magnesium sulphate and 10 *mM* Tris-HCl (pH 7.50) and was separated into two fractions. The K_{part} of the enzyme activity in the first peak was 0.012 whereas that for the retarded fraction was 0.11. Thus it was found that a material with an initial K_{part} of 0.064 in aqueous two-phase systems gives rise to two fractions from the hydrophobic column, having K_{part} values on either side of this value. This suggests that the mixture is resolved according to the same principles as those governing the partition behaviour. It was thus possible to separate strongly

PEG-modified enzyme molecules from unmodified or only weakly modified molecules by this chromatographic technique.

From the studies presented here it seems that use of weak hydrophobic substituents on chromatographic materials yields products with unique properties. It is therefore to be expected that such gels may be useful in the future for resolving mixtures of compounds that are not easily separated today. Separation based on multipoint attachment must, in order to be operable, be based on weak individual interactions. The use of sorbents as discussed in this paper may offer a useful alternative for the separation of particulate structures.

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